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PRINCIPAL INVESTIGATOR: Maria Huacani
D. McDonnell, Ph.D.

CONTRACTING ORGANIZATION: Duke University Medical Center
Durham, North Carolina 27710

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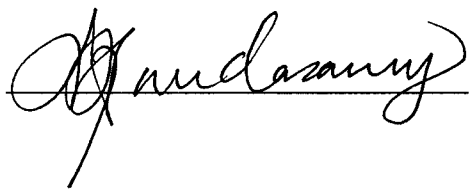

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Sincerely,

Maria R. Huacani

Maria R. Huacani
Principal Investigator
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Maria Huacani
D. McDonnell, Ph.D.

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
Duke University Medical Center
Durham, North Carolina 27710

E-MAIL:
mrh@acpub.duke.edu

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13. ABSTRACT ***(Maximum 200 Words)***

hRPF1/Nedd4 was originally identified in our laboratory as a potentiator of progesterone-receptor transcriptional activity, and subsequently demonstrated to similarly modulate p53-dependent transcription. As a 'hect' E3 ubiquitin ligase, hRPF1/Nedd4's domain structure suggests that it is able to target substrate proteins for ubiquitination. We have been interested in identifying nuclear substrates of hRPF1/Nedd4's ubiquitination activity with a view to understanding the mechanism by which hRPF1/Nedd4 is able to modulate such transcriptional events.

We present compelling evidence that hPRTB, a novel proline-rich protein which colocalizes with splicing machinery in nuclear speckles, is a 'bona fide' nuclear substrate of the WW hect E3 ubiquitin ligase, hRPF1/Nedd4. In addition to providing the first description of a nuclear substrate of mammalian Nedd4, these observations underscore the potential for regulation of splicing proteins by the ubiquitination. Lastly, with the identification of a leucine-rich rev-like nuclear export sequence within hRPF1/Nedd4, we propose that nuclear import/export is an important component of the regulation between the primarily cytoplasmic E3 enzyme, hRPF1/Nedd4 and its nuclear substrate, hPRTB. Thus, we have firmly established a role for hRPF1/Nedd4 within the nucleus, and identified a substrate protein which may explain in part our observed effects upon activated transcription.

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Maria R. Huacani July 22, 2000

PI - Signature Date

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Introduction:

Our interest in proteins which modulate the transcriptional activity of the progesterone receptor (PR) led to the identification of yeast RSP5 and its human homolog, hRPF1/Nedd4 as potentiators of PR-dependent transcription (1). We subsequently observed that hRPF1/Nedd4 has a similar potentiative effect on p53-dependent transcription. Preliminary data indicated that the mechanism of hRPF1/Nedd4's transcriptional effect upon these two nuclear proteins, which are known to participate in the pathogenesis of breast cancer, was likely to be similar. In light of hRPF1/Nedd4's significant sequence homology to the 'hect' class of E3 ubiquitin ligases (2), we initially hypothesized that hRPF1/Nedd4 ubiquitinates and signals the degradation of a protein substrate which is required for PR- and p53-dependent transcription. While additional 'hect' proteins, such as E6-AP have also been demonstrated to participate in nuclear receptor signalling, it is increasingly apparent that these effects are independent of the ubiquitination activity of these enzymes (3). Nonetheless, we have persisted in our search for nuclear substrates of the E3 ubiquitin ligase, hRPF1/Nedd4 in attempt to shed light upon the observed transcriptional effects. Additionally, the proper and faithful recognition of a substrate by an E3 enzyme is a crucial cellular event, and if dysregulated, may lead to cellular transformation (4), (5). Thus, in addition to the goal of understanding a putative role for hRPF1/Nedd4 in transcriptional processes, we have identified substrates of this 'hect' E3 ubiquitin ligase with a view toward furthering our understanding of determinants and regulatory events required for proper E3 ubiquitin ligase/substrate recognition.

Results:

A. Identification of Potential Substrates for hRPF1/Nedd4 Using a Yeast Two Hybrid Screen

A Gal4DBD-hRPF1/Nedd4 fusion was used to screen a human cervical carcinoma (Matchmaker HeLa) cDNA library, and six cDNAs encoding a novel 17 kDa proline-rich protein were isolated as clones which specifically interact with aa. 26-506 of hRPF1/Nedd4). Homology searches using BLAST programs indicated that this 17kDa protein is identical to KIA0058, a cDNA isolated from a myeloid cell line, KG-1. We and others have termed this human cDNA, hPRTB (Figure 1A) based upon its high amino acid identity with mouse PRTB (Proline-rich transcript, brain expressed), which was isolated in a gene trapping screen as a transcript expressed in the developing mouse inner ear (6). hPRTB has no known motifs or domains when examined by PROSITE, and its most notable feature is its proline-rich composition (18%), as suggested by its name.

To independently verify that hPRTB does indeed interact with full length hRPF1/Nedd4, we assayed the ability of in vitro translated, ^{35}S-methionine labelled myc-hPRTB to interact with recombinant GST-fusions of hRPF1/Nedd4. Myc-hPRTB was able to interact with full length hRPF1/Nedd4 or yRSP5, a yeast homolog which has similar domain structure to hRPF1 (containing only three WW domains). The WW domains of

hRPF1/Nedd4 were sufficient for interaction with hPRTB, while neither the 'hect' domain nor the C2 domain resulted in any detectable interaction (Figure 1B). WW domains are predicted to interact with proline containing consensus sequences which are either PPXY, PPLP, PGM (7), (8). Examination of the proline-rich regions within hPRTB revealed a consensus 'PPAY' motif located in the central portion of the protein. With the prediction that this conserved motif may mediate hPRTB's interaction with hRPF1/Nedd4, we substituted the second proline and subsequent tyrosine with alanines and assayed ability of this 'PY' mutant to interact with hRPF1/Nedd4. The two amino acid substitution within the 'PPAY' motif in hPRTB was able to disrupt the ability of hPRTB to bind to hRPF1/Nedd4 (Figure 1B), further demonstrating that the WW domains of hRPF1/Nedd4 directly interact with the 'PPAY' motif of hPRTB.

B. hPRTB is a Substrate of the WW Hect E3 Ubiquitin Ligase, hRPF1/Nedd4

Though we have identified hPRTB as a protein which specifically binds to hRPF1/Nedd4, we were most interested to analyze its ability to serve as a substrate for E3 ubiquitin ligase activity. Using a standard *in vitro* ubiquitination assay (9), we observed ubiquitination of hPRTB when assayed in the presence of purified hRPF1/Nedd4 (whect) or yRSP5, but not the hect E3 ligase E6-AP which lacks WW domains in its amino terminus (Figure 2A). Mutation of two key residues within the 'PPAY' motif of hPRTB was able to completely abrogate ubiquitination of hPRTB by either hRPF1/Nedd4 (whect) or yeast RSP5. Thus, we have shown a clear correlation between binding of hPRTB to hRPF1/Nedd4 and its ability to be ubiquitinated *in vitro*.

As hPRTB is an efficient substrate *in vitro*, we next demonstrated that hPRTB is a physiological substrate for ubiquitination. Briefly, we transfected HeLa cells with expression plasmids for a His-tagged ubiquitin construct as well as a myc-tagged hPRTB expression plasmid. In theory, any cellular ubiquitination substrate should have a population of protein which is modified by this His-ubiquitin, which can be isolated on Ni-NTA resin. His-ubiquitin conjugates were detected only upon cotransfection of His-ubiquitin and wild type myc-hPRTB (Figure 2B). Transfection of either myc-hPRTB alone (lane 4) or cotransfection of myc-hPRTB-'PY'mut and His-ubiquitin (lane 6) were unable to produce His-ubiquitin hPRTB conjugates, although lanes 1-3 verify that all proteins were expressed in HeLa cellular lysates. Our observation that myc-hPRTB-'PY'mut appears to accumulate to higher steady state levels than myc-PRTB (compare lane3 with lanes 1-2, Figure 2B) led us to next compare the half-life of wild type and PY mutant hPRTB proteins.

Using pulse-chase analysis, we analyzed the half-lives of myc-hPRTB or the myc-hPRTB 'PY' mutant in Hela cells. Wild type hPRTB has a significantly shorter half life than the hPRTB 'PY' mutant which blocks ubiquitination. The average and quantitation of several independent experiments, indicates that mutation of two key residues within the 'PPAY' motif of hPRTB results in a 3.5 fold increase in half-life of hPRTB from about 2 hours to 7 hours. Cumulatively, these observations are compelling evidence that hPRTB is a physiological substrate of an endogenous WW-hect E3 ubiquitin ligase, such as hRPF1/Nedd4.

Importantly, we last sought to establish that exogenous hRPF1/Nedd4 was able to alter the ubiquitin-dependent degradation of hPRTB. Pulse-chase analysis of hPRTB protein levels in the absence or presence of cotransfected E3 ubiquitin ligase enzyme, resulted in accelerated degradation of PRTB in samples containing transiently transfected hRPF1/Nedd4, but not in samples transfected with the catalytic mutant, hRPF1/Nedd4-C867A, or an empty expression plasmid (Figure 3A). The half-life of PRTB in the presence of overexpressed hRPF1/Nedd4 was decreased by at least 100 minutes, when compared to samples containing only endogenous hRPF1/Nedd4 (Figure 3B). Thus, we have identified a nuclear speckle associated protein, hPRTB, as a substrate of the E3 WW-hect ubiquitin ligase, hRPF1/Nedd4 within cells.

C. Relationship between Nuclear hPRTB and Cytoplasmic hRPF1/Nedd4 in Cultured Cells

hPRTB Colocalizes with Splicing Factors in Nuclear Speckles

With the goal of obtaining clues about the function of this novel proline-rich substrate of hRPF1/Nedd4, we fused hPRTB to the EGFP protein and analyzed its subcellular localization using fluorescence confocal microscopy. Both EGFP-hPRTB and the 'PY' mutant have identical fluorescence patterns and are localized to the nucleus in a discrete speckled pattern (Figure 4). We were intrigued by our observation of its localization in spots in the nucleus, reminiscent of nuclear speckles which are enriched in splicing factors and contain a population of hyperphosphorylated RNA polymerase II (10). It is increasingly understood that RNA transcription and splicing are coordinated processes, and recent observations physically link the CTD of the large subunit of RNA polymerase II with RNA processing and splicing machinery (11), (12). As the large subunit of RNA polymerase II is a known ubiquitination substrate of the yeast RSP5 (9),it is possible that hPRTB, a potential human ubiquitination substrate of a RSP5-like E3 ligase, localizes to the same place in the nucleus as RNA polymerase II. Using fluorescent confocal microscopy, we demonstrate that hPRTB colocalizes with the splicing factor, SC35, in nuclear speckles (Figure 4), suggesting that hPRTB may have a role in the transcription and/or splicing of RNA transcripts. Thus in addition to identifying a potential substrate of hRPF1/Nedd4 which is found in the nucleus, we have localized hPRTB to a subcellular localization similar to that of another WWhect E3 substrate, RNA polymerase II.

hRPF1/Nedd4 Contains a rev-like Nuclear Export Sequence

To further confirm our observations that hRPF1/Nedd4 is able to target the nuclear protein hPRTB for ubiquitination and degradation, we lastly pursued a series of experiments to understand the mechanism by which a primarily cytoplasmic E3 enzyme, hRPF1/Nedd4, is able to modify the nuclear protein, hPRTB.

While hRPF1/Nedd4 has been reported to contain a bipartite nuclear localization signal between amino acids 534-550, cumulative observations suggest that Nedd4 is a primarily cytoplasmic protein (13), (14). In attempt to artificially place hRPF1/Nedd4 into the nuclear compartment of cells, we fused a strong SV40 nuclear localization signal (NLS)

to the amino terminus of hRPF1/Nedd4. When cellular localization of this exogenous SV40NLS-RPF1/Nedd4 was assayed, there was surprisingly little to no nuclear staining despite obvious overexpression as assayed by Western blot analysis, raising the possibility that hRPF1/Nedd4 contains a strong nuclear export signal (NES).

Many nuclear proteins undergo nuclear export dependent upon the CRM1-dependent export pathway (15), (16), (17). To assay if indeed hRPF1/Nedd4 might be a substrate of nuclear export, we utilized the drug leptomycin B (LMB), a specific inhibitor of CRM-1 dependent export (18). A population of both wild-type and catalytically inactive hRPF1/Nedd4 was localized within the nucleus after treatment with LMB, indicating that this WWhect E3 ubiquitin ligase, or a complex containing this protein, is a substrate of CRM-1 dependent nuclear export.

Given the data of others that a portion of hRPF1/Nedd4 encoding aa. 404-900 is localized primarily within the nucleus (13) while full length constructs are cytoplasmic, we next created a series of N-terminal deletion constructs in attempt to map the region of hRPF1/Nedd4 which may be responsible for cytoplasmic localization. Full length hRPF1/Nedd4, as well as aa.173-900 and aa. 293-900 localized primarily to the cytoplasm, while proteins encoding aa. 309-900 and 404-900 were present in both the cytoplasm and the nucleus. Thus, a sequence within amino acids 293 and 309 of hRPF1/Nedd4 is responsible for the steady state localization of Nedd4 in the cytoplasm.

With the hypothesis that a nuclear export sequence within hRPF1/Nedd4 may explain its localization, we compared amino acids 293-309 of hRPF1/Nedd4 with the leucine-rich consensus for rev-like nuclear export. Indeed, amino acids 297-307 of hRPF1/Nedd4 contain sequence identity with this nuclear export sequence (NES) consensus, and share significant homology with the NES sequences found in other proteins such as PKI, HIVrev, human p53 and rex (Figure 5A) (19), (20), (15). To prove that this sequence within hRPF1/Nedd4 is able to act as a NES, we mutated L305A and I307A and assayed for the cellular localization of this pututive NES mutant. Mutation of these two conserved amino acids within the export sequence resulted in the nuclear and cytoplasmic localization of myc-hRPF1/Nedd4-C867A, as compared to the primarily cytoplasmic localization of myc-hRPF1/Nedd4-C867A (Figure 5B) or myc-RPF1. Interestingly, when the NES mutant was created in the context of a wild type hRPF1/Nedd4, extremely low quantities of exogenous protein were detected by western blot, and these levels were below the limit of detection of fluorescence microscopy techniques. Nonetheless, these observations cumulatively demonstrate that amino acids 297-309 mediate the CRM1-dependent nuclear export of hRPF1/ Nedd4.

Conclusions:

Cumulatively, we present in this study compelling evidence that hPRTB, a novel proline-rich protein which colocalizes with splicing machinery in nuclear speckles, is a 'bona fide' nuclear substrate of the WW hect E3 ubiquitin ligase, hRPF1/Nedd4. In addition to providing the first description of a nuclear substrate of mammalian Nedd4, these observations underscore the potential for regulation of splicing proteins by the ubiquitination. Lastly, with the identification of a leucine-rich rev-like nuclear export sequence within hRPF1/Nedd4, we propose that nuclear import/export is an important component of the regulation between the primarily cytoplasmic E3 enzyme, hRPF1/Nedd4 and its nuclear substrate, hPRTB.

Though our previous analysis of the relationship between hRPF1/Nedd4 and PR- and p53-dependent transcription has suggested an indirect effect upon PR- and p53-dependent transcription (reported in Progress Report of July 1999), the work described above suggests a mechanism by which hRPF1/Nedd4 may directly impact certain post-transcriptional events. It is conceivable that hRPF1/Nedd4 may ultimately modulate the stability or type of RNA transcripts produced within a cell via degradation of putative RNA processing proteins such as hPRTB. We anticipate that additional studies aimed at clarifying the potential role of ubiquitination in RNA processing will be of significant importance in furthering our understanding of the regulation of activated transcription.

Key Research Accomplishments

(Accomplishments since last Progress Report, July 1999, indicated in bold)

- Completion of yeast two-hybrid screen with identification of 6 proteins which specifically bind to the amino terminal 'substrate binding domain' of hRPF1/Nedd4

- Confirmation of yeast two-hybrid interactions using GST-pulldown interaction assays

- Cloning of full-length cDNAs for two hRPF1/Nedd4 interacting proteins

- **Colocalization of hPRTB with splicing machinery in nuclear speckles**

- Creation of recombinant baculovirus for hRPF1/Nedd4 expression; purification of hRPF1/Nedd4 and hRPF1/Nedd4-C867A from baculovirus/SF9 cells

- Establishment of *in vitro* ubiquitination assay using yRSP5 or hRPF1/Nedd4 as E3 ubiquitin ligase

- Identification and creation of amino acid substitutions in substrate proteins which abrogate ubiquitination

- **Isolation of *in vivo* ubiquitin conjugates of hPRTB within cells**

- **Pulse chase demonstration of degradation of hPRTB dependent upon intact 'PPXY' motif**

- **Observed increased degradation of hPRTB in presence of exogenous hRPF1/Nedd4 (but not catalytically inactive hRPF1/Nedd4-C867A)**

- **Deletion mapping and identification of a nuclear export sequence between aa. 297-307 of hRPF1/Nedd4**

A.

1 MNSKGQYPTQPTYPVQPPGNPVYPQ
TLHLPQAPPYTDAPPAYSELYRPSF
VHPGAATVPTMSAAFPGASLYLPMA
QSVAVGPLGSTIPMAYYPVGPIYPP
GSTVLVEGGYEAGARFGAGATAGNI
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QRKGNFFMGGSDGGYTIW 151

B.

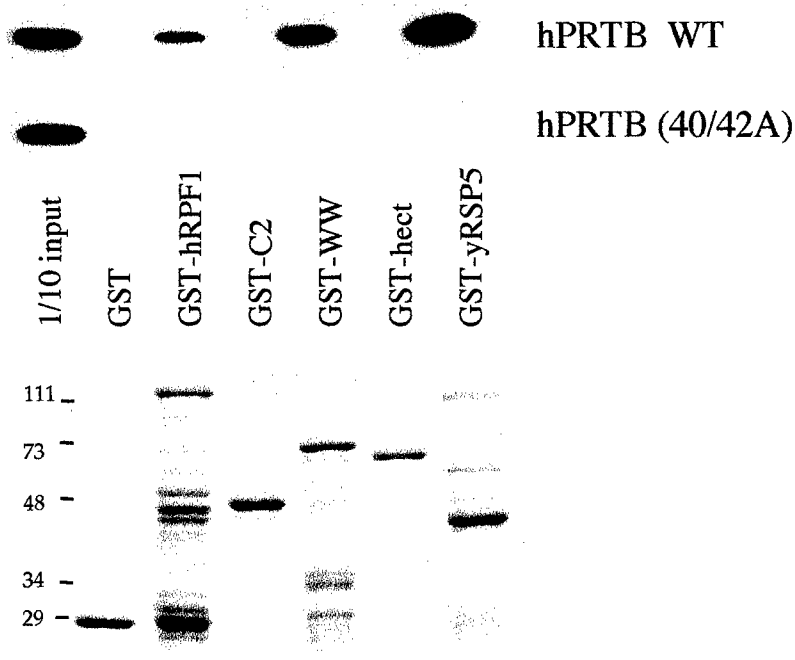


Figure 1: A. Protein sequence for human PRTB (proline transcript brain expressed). The 'APPAY' motif, which is mutated in studies to follow, is indicated by the outlined rectangle. The numerous proline residues are highlighted by bold type.

B. GST-pulldown interactions between GST-hRPF1/Nedd4 and myc-hPRTB. GST alone, GST fusions of hRPF1/Nedd4 (or indicated portions), or GST-yRSP5 were immobilized on glutathione sepharose and incubated with ^{35}S-labelled myc-PRTB or myc-PRTB(PY mutant) overnight at 4°C. Bead bound proteins were extensively washed prior to SDS-PAGE and autoradiography. Fusion proteins used for GST-pulldown interaction studies are shown in lower panel. Approximately equivalent microgram amounts of GST or GST-fusion proteins were resolved by SDS-PAGE and detected by Coomassie blue staining.

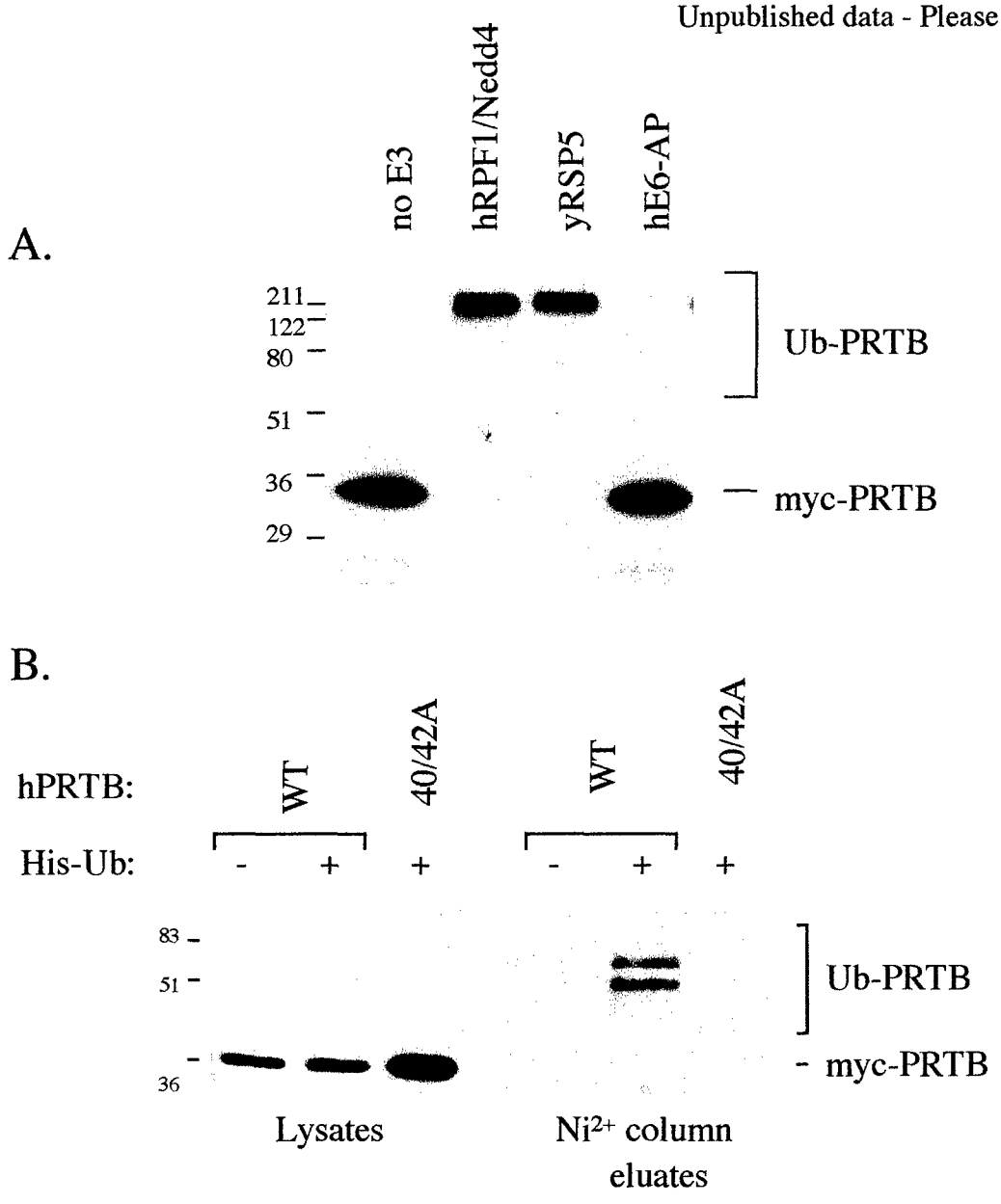
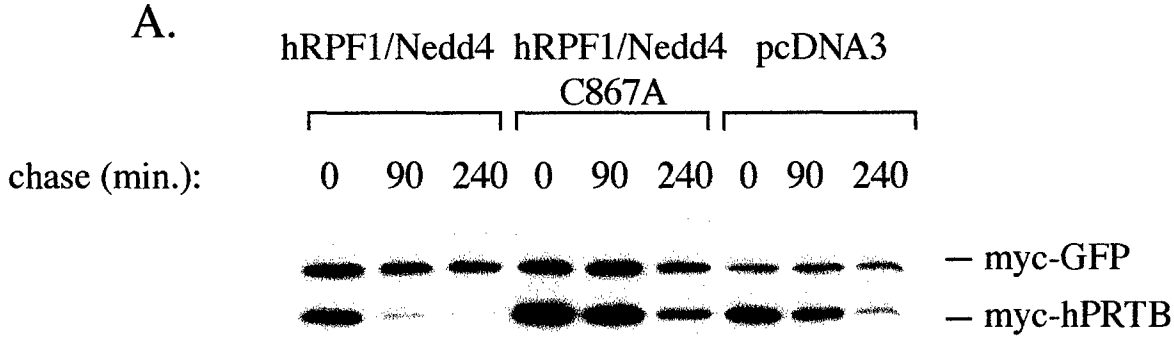


Figure 2: A. hRPF1/Nedd4 ubiquitinates hPRTB *in vitro*. 35S-labelled myc-hPRTB was incubated with purified hRPF1/Nedd4 (whect), yRSP5 or hE6-AP in the presence of ATP, ubiquitin, and bacterially expressed E1 and E2 (UbcH5B) enzymes.
B. His-ubiquitin conjugates of hPRTB isolated from Hela cells. Hela cells were transfected with expression plasmids for His-ubiquitin and myc-hPRTB or myc-hPRTB(PY mutant). Forty hours post transfection, denatured lysates were prepared (lanes 1-3) and His-conjugates were purified on nickel resin (lanes 4-6). Myc-hPRTB or myc-hPRTB his-ubiquitin conjugates were detected by western blot analysis using an antibody directed against c-myc (9E10).

A.



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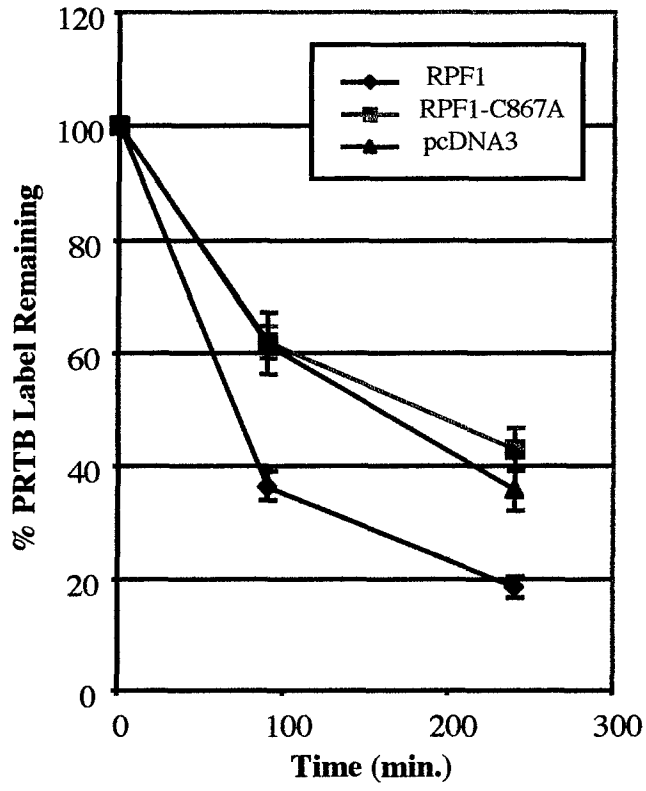
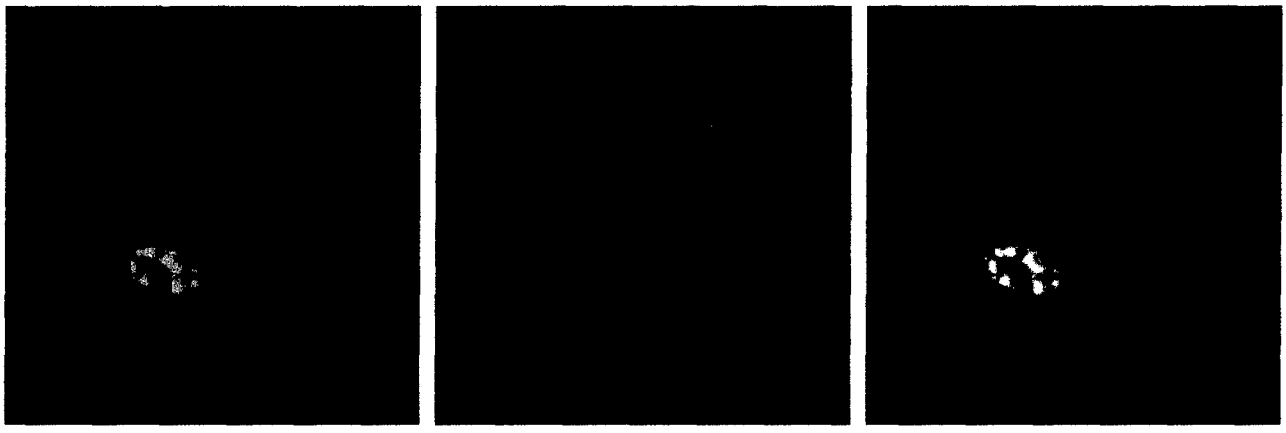


Figure 3: A. hRPF1/Nedd4 but not its C867A catalytic point mutant accelerates degradation of PRTB. Hela cells were cotransfected with myc-PRTB, the internal control myc-EGFP, and pcDNA3, hRPF1/Nedd4 or hRPF1/Nedd4-C867A. Cells were subsequently radioactively labelled, and chased in 'cold' media. Lysates from indicated time points were immunoprecipitated using a c-myc antibody (9E10) and analyzed by SDS-PAGE followed by autoradiography.

B. Half-life of hPRTB is decreased in the presence of exogenous hRPF1/Nedd4. Results from three independent experiments were quantitated using a phosphorimager, normalized against an internal myc-GFP control and expressed as percentage of labelled hPRTB protein at time zero.



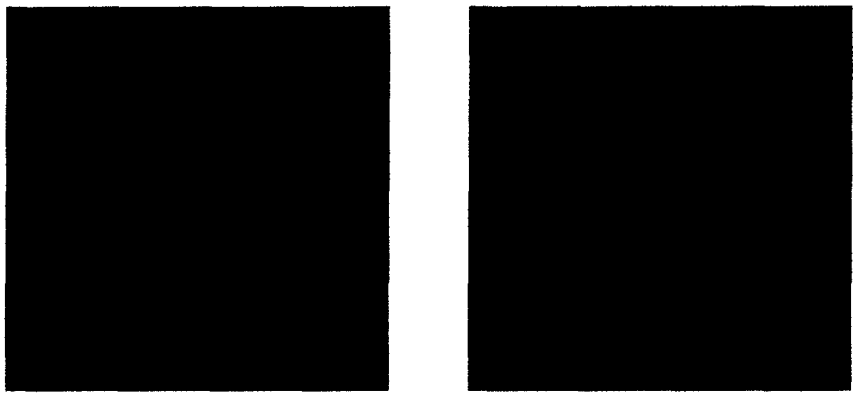
GFP-hPRTB SC35 both

Figure 4: hPRTB colocalizes with splicing factor SC35 in nuclear speckles. The EGFP-NI vector was used to express hPRTB as a N-terminal GFP fusion in Hela cells. Twenty-four hours post transfection cells were plated onto glass coverslips, allowed to attach, and subsequently fixed, permeabilized and incubated with a monoclonal antibody which recognizes the nuclear speckle-associated splicing factor, SC35. GFP or mouse-Texas red fluorescence was detected using confocal microscopy.

A.

	297									307
hRPF1/Nedd4	**L**	**A**	**E**	**E**	**L**	**N**	**A**	**R**	**L**	**T**	**I**
PKI	**L**	**A**	**L**	**K**	**L**	**-**	**A**	**G**	**L**	**D**	**I**
HIVrev	**L**	**P**	**-**	**P**	**L**	**-**	**E**	**R**	**L**	**T**	**L**
p53 human	**M**	**F**	**R**	**E**	**L**	**N**	**E**	**A**	**L**	**E**	**L**
Rex	**L**	**S**	**A**	**Q**	**L**	**Y**	**S**	**S**	**L**	**S**	**L**
Consensus	**L**	**x**	**x**	**x**	**L**	**x**	**x**	**x**	**L**	**x**	**L/I**

B.



myc-RPF1-C867A

myc-RPF1-C867A mutNES

Figure 5: A. hRPF1/Nedd4 contains a consensus rev-like nuclear export sequence (NES). Amino acids 297-307 are aligned with the export sequences of PKI, HIVrev, human p53, and rex. Conserved leucines within the consensus NES sequence are highlighted in blue.

B. Mutation of the NES result in a steady-state population of hRPF1/Nedd4-CA in the nucleus. Two conserved residues within the NES of hRPF1/Nedd4 were substituted with alanine (L305A, I307A) within the context of the C867A catalytic mutant of myc-tagged hRPF1/Nedd4. cDNAs were transiently transfected into Hela cells and cells prepared for immunofluorescence as previously described.

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REPORTABLE OUTCOMES

PUBLICATIONS:

Sylvie L. Beaudenon, **Maria R. Huacani**, Guangli Wang, Donald P. McDonnell, and Jon M. Huibregtse. (1999) The Rsp5 ubiquitin-protein ligase mediates DNA damage-induced degradation of the large subunit of RNA polymerase II in *Saccharomyces cerevisiae*. *Molecular and Cellular Biology* **19**:6972-6979.

John D. Norris, Lisa A. Paige, Dale J. Christensen, Ching-yi Chang, **Maria R. Huacani**, Daju Fan, Paul T. Hamilton, Dana M. Fowlkes, Donald P. McDonnell. (1999) Peptide Antagonists of the Human Estrogen Receptor. *Science* **285**: 744-746.

CONFERENCE PRESENTATIONS AND POSTERS:

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Peptide Antagonists of the Human Estrogen Receptor

John D. Norris,[1] Lisa A. Paige,[2] Dale J. Christensen,[2] Ching-Yi Chang,[1] Maria R. Huacani,[1] Daju Fan,[1] Paul T. Hamilton,[2] Dana M. Fowlkes,[2] Donald P. McDonnell[1]*

Estrogen receptor α transcriptional activity is regulated by distinct conformational states that are the result of ligand binding. Phage display was used to identify peptides that interact specifically with either estradiol- or tamoxifen-activated estrogen receptor α. When these peptides were coexpressed with estrogen receptor α in cells, they functioned as ligand-specific antagonists, indicating that estradiol-agonist and tamoxifen–partial agonist activities do not occur by the same mechanism. The ability to regulate estrogen receptor α transcriptional activity by targeting sites outside of the ligand-binding pocket has implications for the development of estrogen receptor α antagonists for the treatment of tamoxifen-refractory breast cancers.

About 50% of all breast cancers express the estrogen receptor α (ERα) protein and recognize estrogen as a mitogen (*1*). In a subpopulation of these tumors, antiestrogens, compounds that bind ER and block estrogen action, effectively inhibit cell growth. In this regard, the antiestrogen tamoxifen has been widely used to treat ER-positive breast cancers (*2*). Although antiestrogen therapy is initially successful, most tumors become refractory to the antiproliferative effects of tamoxifen within 2 to 5 years. The mechanism by which resistance occurs is controversial; however, it does not appear to result as a consequence of ER mutations or altered drug metabolism (*3*). It may relate instead to the observation that tamoxifen is a selective estrogen receptor modulator (SERM), functioning as an ER agonist in some cells and as an antagonist in others (*4*). Consequently, the ability of tumors to switch from recognizing tamoxifen as an antagonist to recognizing it as an agonist has emerged as the most likely cause of resistance. Upon binding ER, both estradiol and tamoxifen induce distinct conformational changes within the ligand-binding domain (*5*). The tamoxifen-induced conformational change may expose surfaces on the receptor that allow it to engage the general transcription machinery. We used phage display to identify specific peptides that interacted with the estradiol- and tamoxifen-ER complexes and used these peptides to show that estradiol and tamoxifen manifest agonist activity by different mechanisms.

Affinity selection of phage-displayed peptide libraries was performed to identify peptides that could interact specifically with the agonist [17β-estradiol (estradiol) or 4-OH tamoxifen (tamoxifen)], activated ERα, or ERβ (*6*). Representative peptides from each of four classes presented in this study are shown in Fig. 1A. Several peptides that were isolated with estradiol-activated ERα (represented by α/β I) contained the Leu-X-X-Leu-Leu motif found in nuclear receptor coactivators (*7*). α II was isolated with either estradiol- or tamoxifen-activated ERα. Two classes of peptides, α/β III and α/β V, that interact specifically with tamoxifen-activated ERα and ERβ, respectively, were identified. The α/β V peptide was subsequently shown to interact with tamoxifen-activated ERα (*6*). Several additional peptides homologous to α/β V were identified. A BLAST search of the National Center for Biotechnology Information database with the derived consensus of the α/β V peptide class revealed that the yeast protein RSP5 and its human homolog, receptor potentiating factor (RPF1), both contain sequences homologous to α/β V. These proteins were previously shown to be coactivators of progesterone receptor B (PRB) transcriptional activity (*8*).

Peptide-peptide competition studies were performed with time-resolved fluorescence (TRF) to determine if the α II, α/β III, and α/β V peptides were binding the same or distinct "pockets" on the tamoxifen-ERα complex (*9*). The α/β III and α/β V peptides cross compete, and at equimolar peptide concentrations, 50% inhibition is observed (Fig. 1B). This result indicates that these two peptides bind to the same or overlapping sites on ERα. We believe that the α II peptide binds to a unique site as its binding was not competed by α/β V and only 50% inhibited by a 10-fold excess of the α/β III peptide.

We next assessed whether the peptides interacted with ERα in vivo using the mammalian two-hybrid system (*10*). The α/β I peptide interacted with ERα in the presence of the agonist estradiol but not the SERMs tamoxifen, raloxifene, GW7604, idoxifene, and nafoxidine or the pure antagonist ICI 182,780 (Fig. 2). The failure of antiestrogen-activated ERα to interact with the α/β I peptide is consistent with previ-

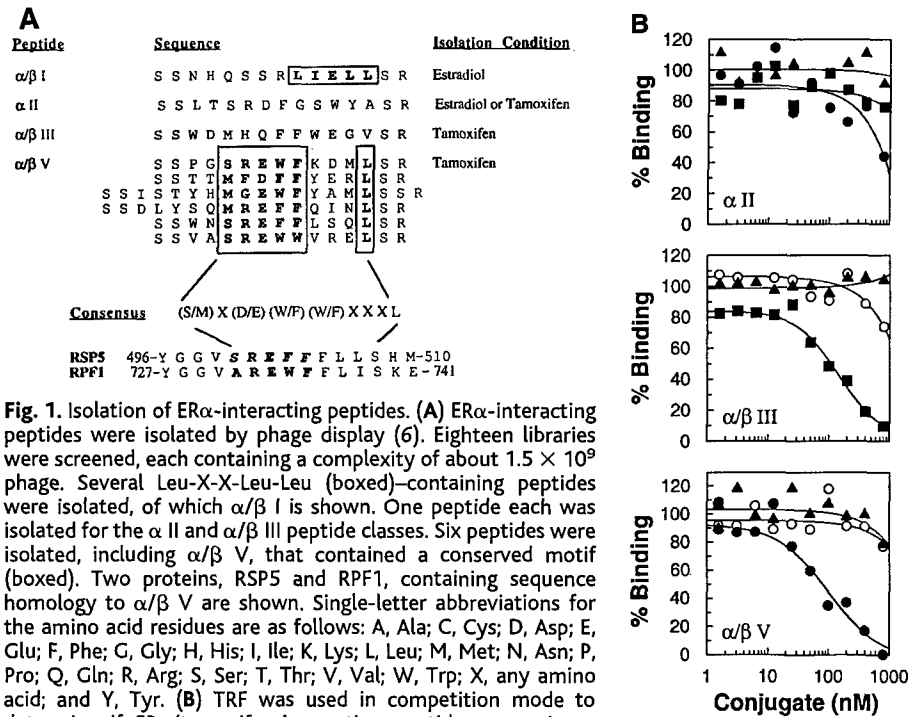

Fig. 1. Isolation of ERα-interacting peptides. (**A**) ERα-interacting peptides were isolated by phage display (*6*). Eighteen libraries were screened, each containing a complexity of about 1.5×10^9 phage. Several Leu-X-X-Leu-Leu (boxed)–containing peptides were isolated, of which α/β I is shown. One peptide each was isolated for the α II and α/β III peptide classes. Six peptides were isolated, including α/β V, that contained a conserved motif (boxed). Two proteins, RSP5 and RPF1, containing sequence homology to α/β V are shown. Single-letter abbreviations for the amino acid residues are as follows: A, Ala; C, Cys; D, Asp; E, Glu; F, Phe; G, Gly; H, His; I, Ile; K, Lys; L, Leu; M, Met; N, Asn; P, Pro; Q, Gln; R, Arg; S, Ser; T, Thr; V, Val; W, Trp; X, any amino acid; and Y, Tyr. (**B**) TRF was used in competition mode to determine if ERα/tamoxifen-interacting peptides recognize a common site on ERα (*9*). The peptide conjugate used for detection is indicated in each graph with the competing peptides as follows: ▲, no competitor; ○, α II; ●, α/β III; and ■, α/β V.

[1]Duke University Medical Center, Department of Pharmacology and Cancer Biology, Durham, NC 27710, USA. [2]Novalon Pharmaceutical Corporation, 4222 Emperor Boulevard, Suite 560, Durham, NC 27703, USA.
*To whom correspondence should be addressed. E-mail: mcdon016@acpub.duke.edu

ous studies that predict that the molecular mechanism of antagonism results from a structural change in the receptor ligand-binding domain that prevents coactivators from binding (*5*). α II interacted with the receptor in the presence of all modulators tested, with the unliganded (vehicle) and ICI 182,780–bound receptors showing the least binding activity. α/β III and α/β V interacted almost exclusively with the tamoxifen-bound ERα. ERα did not interact with the Gal4 DNA-binding domain (DBD) (control) alone in the presence of any modulators tested. Further studies indicated that binding of α II, α/β III, and α/β V occurs within the hormone-binding domain between amino acids 282 and 535 (*11*) and, unlike binding of α/β I, does not require a functional activation function 2 (AF-2) (www.sciencemag.org/feature/data/1039590.shl). These data indicate that SERMs induce different conformational changes in ERα within the cell and firmly establish a relation between the structure of an ERα-ligand complex and function.

When we examined the specificity of interaction between the peptides and heterologous nuclear receptors, we found, as expected, that the α/β I peptide interacted with ERβ, PRB, and the glucocorticoid receptor (GR) when bound by the agonists estradiol, progesterone, and dexamethasone, respectively (Fig. 3, A, B, and C). The α/β V peptide interacted with tamoxifen-bound ERβ and unexpectedly with PRB in the presence of the antagonists RU 486 or ZK 98299 (Fig. 3, A and B). The α/β V peptide, however, did not interact with the GR when bound by RU 486 or ZK 98299. α II and α/β III peptides failed to interact with ERβ, PRB, or GR.

We next tested the ability of the peptide-Gal4 fusion proteins to inhibit ERα transcriptional activity. Tamoxifen displayed partial agonist activity when analyzed with the ER-responsive complement 3 (C3) promoter in HepG2 cells (Fig. 4A). This activity can reach 35% of that exhibited by estrogen and is mediated by three nonconsensus estrogen response elements (EREs) located in the C3 promoter (*12*). When expressed in this system, the α/β I and α II peptides inhibited the ability of estradiol to activate transcription up to 50% and 30%, respectively (Fig. 4B). Two copies of the Leu-X-X-Leu-Leu sequence found in α/β I enhanced the inhibitory effect of this peptide and blocked estradiol-mediated transcription by about 90% (*13*). The inability of α/β III and α/β V to block estradiol-mediated transcription correlates well with their inability to bind the receptor when bound by agonist. Expression of α II, α/β III, and α/β V peptides blocked the partial agonist activity of tamoxifen (Fig. 4C). α II and α/β V were the most efficient disrupters of tamoxifen-mediated transcription, inhibiting this activity by about 90%. All peptide-Gal4 fusion proteins were expressed at similar levels, indicating that the relative differences in inhibition are not due to peptide stability (*11*). We also demonstrated that receptor stability and DNA binding are not affected by peptide expression (*11*). As expected, α/β I was unable to inhibit tamoxifen-mediated transcription. These findings are in agreement with the binding characteristics of these peptides and suggest that the pocket or pockets recognized by α II, α/β III, and α/β V are required for tamoxifen partial agonist activity. Although α/β V was shown to interact with PRB when bound by RU 486 (Fig. 3B), it was unable to block the partial agonist activity mediated by PRB/RU 486 (*11*). This result suggests that ERα/tamoxifen and PRB/RU 486 partial agonist activities are manifested differently. However, because α/β V was selected against ERα, this peptide may not bind PRB with high enough affinity to permit it to be useful as a PRB peptide antagonist.

Finally, we examined the ability of these peptides to inhibit ER transcriptional activity mediated through AP-1–responsive genes. This pathway has been proposed to account for some of the cell-specific agonist activity of tamoxifen (*14*). Both estradiol and tamoxifen activated transcription from the AP-1–responsive collagenase reporter gene, pCOL-Luc (Fig. 4D). This activity is manifest in the absence of an ERE and is believed to occur through a mechanism involving an interaction between ERα and the promoter-bound AP-1 complex (*14*). Regardless of the mechanism, each peptide was able to inhibit ERα-mediated transcriptional activity in a manner that reflected its ability to interact with the receptor in a ligand-dependent manner (Fig. 4E).

The mechanism by which tamoxifen manifests SERM activity is not yet known. Evidence presented in this study suggests that the tamoxifen-bound receptor exposes a binding site that is occupied by a coactivating protein not primarily used by the estradiol-activated receptor. The α II peptide, which interacts with both estradiol- and tamoxifen-bound receptors, inhibits the partial agonist activity of tamoxifen efficiently, while minimally affecting estradiol-mediated transcription. This result suggests that this site, although crucial for tamoxifen-mediated transcription, is dispensable for estrogen action. In addition, the ability of α/β III and α/β V to bind tamoxifen-specific surfaces and inhibit tamoxifen-mediated partial agonist activity suggests that these peptides may potentially recognize a protein contact site on ER that is critical for this activity. In this regard, we can

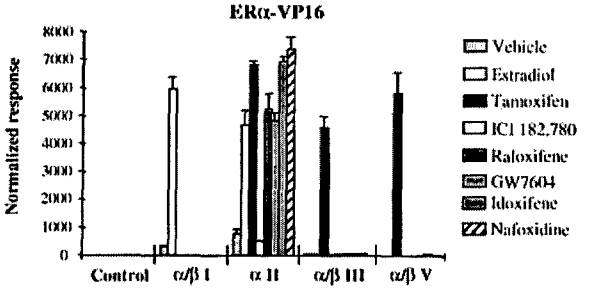

Fig. 2. ERα-peptide interactions in mammalian cells. The coding sequence of a peptide representative from each class identified was fused to the DBD of the yeast transcription factor Gal4. HepG2 cells were transiently transfected with expression vectors for ERα-VP16 and the peptide-Gal4 fusion proteins. In addition, a luciferase reporter construct under the control of five copies of a Gal4 upstream enhancer element was also transfected along with a pCMV–β-galactosidase (β-Gal) vector to normalize for transfection efficiency. Transfection of the Gal4 DBD alone is included as control. Cells were then treated with various ligands (100 nM) as indicated and assayed for luciferase and β-Gal activity. Normalized response was obtained by dividing the luciferase activity by the β-Gal activity. Transfections were performed in triplicate, and error bars represent standard error of the mean (SEM). Triplicate transfections contained 1000 ng of ERα-VP16, 1000 ng of 5× Gal4-tata-Luc, 1000 ng of peptide-Gal4 fusion construct, and 100 ng of pCMV–β-Gal (*10*).

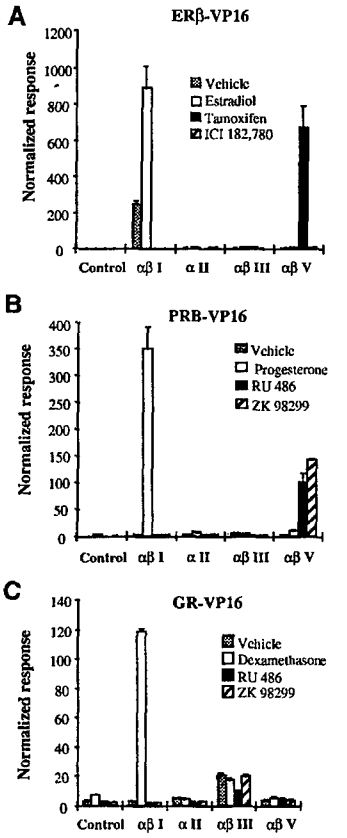

Fig. 3. Specificity of nuclear receptor–peptide interactions. Two-hybrid experiments were performed as in Fig. 2 between peptide-Gal4 fusion proteins and either (**A**) ERβ-VP16, (**B**) PRB-VP16, or (**C**) GR-VP16 (*15*). RU 486 and ZK 98299 are pan-antagonists of PRB and GR.

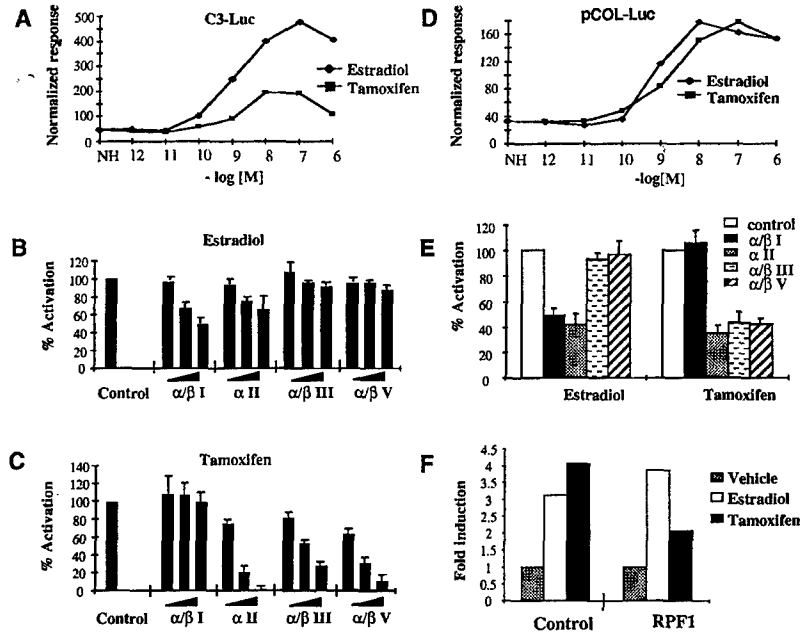

Fig. 4. Disruption of ERα-mediated transcriptional activity. **(A)** HepG2 cells were transfected with the estrogen-responsive C3-Luc reporter gene (*12*) along with expression vectors for ERα (*16*) and β-Gal and normalized as in Fig. 2. Cells were induced with either estradiol or tamoxifen as indicated and analyzed for luciferase and β-Gal activity. NH, no hormone. **(B)** HepG2 cells were transfected as in (A) except that expression vectors for peptide-Gal4 fusions were included as indicated. Control represents the transcriptional activity of estradiol (10 nM)–activated ERα in the presence of the Gal-4 DBD alone and is set at 100% activity. Increasing amounts of input plasmid for each Gal4-peptide fusion are also shown (▲) with the resulting transcriptional activity presented as percentage of activation of control. Data are averaged from three independent experiments (each performed in triplicate) with error bars representing SEM. Triplicate transfections contained 1000 ng of C3-Luc, 1000 ng of ERα expression vector, 100 ng of pCMV–β-Gal, and either 100, 500, or 1000 ng of peptide-Gal4 fusion construct. **(C)** Same as in (B) except that 4-OH tamoxifen (10 nM) was used to activate the receptor. **(D)** HepG2 cells were transfected with the AP-1–responsive collagenase reporter gene construct (pCOL-Luc) (*12*) and expression vectors for ERα and β-Gal. Cells were then induced with either estradiol or tamoxifen as indicated. **(E)** Same as (D), except that peptide-Gal4 fusion constructs were also transfected as indicated. Control represents the transcriptional activity of either estradiol- or tamoxifen (100 nM)–activated ER in the presence of the Gal4 DBD alone and is set at 100% activity. The transcriptional activity of estradiol and tamoxifen is shown in the presence of each Gal4-peptide fusion with the resulting transcriptional activity presented as percentage of activation of control. Triplicate transfections contained 1000 ng of pCOL-Luc, 1000 ng of ERα expression vector, 1000 ng of peptide-Gal4 fusion construct, and 100 ng of pCMV–β-Gal. Data are presented as in (B) and (C). **(F)** HeLa cells were transfected with the 1X-ERE-tata-Luc reporter gene along with expression vectors for ERα, β-Gal, and either RPF1 (pCDNA3-RPF1) or control vector [pcDNA3 (Invitrogen, Carlsbad, CA)]. Cells were induced with ligand (10 nM) as indicated. Data are presented as fold induction, which represents the ratio of ligand induced versus vehicle for each transfection.

demonstrate that, similar to α/β V, overexpression of RPF1 specifically represses tamoxifen-mediated partial agonist activity (Fig. 4F). However, the physiological importance of this activity remains to be determined. In summary, we have identified a series of peptide antagonists of ERα and hence validated additional target sites other than the ligand-binding pocket for drug discovery.

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6. Phage display was performed as described [L. A. Paige *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* **96**, 3999 (1999)]. Immulon 4 96-well plates (Dynex Technologies, Chantilly, VA) were coated with streptavidin in $NaHCO_3$ buffer (pH 8.5) at 4°C for about 18 hours. Wells were blocked with bovine serum albumin (BSA) and then washed with TBST [10 mM tris-HCl (pH 8.0), 150 mM NaCl, and 0.05% Tween 20], and 2 pmol of biotinylated vitellogenin ERE was then added per well. Plates were washed with TBST, 3 pmol of baculovirus-purified ERα or ERβ (Pan Vera, Madison, WI) was then added, and plates were incubated at room temperature for 1 hour. Hormone was then added (1 μM) along with phage library (containing about 1.5×10^9 phage) in TBST and incubated at room temperature for 1 hour. Nonbinding phage were removed by washing with TBST. Bound phage were eluted in prewarmed (50°C) 50 mM glycine-HCL (pH 2.0). Eluent was neutralized by the addition of 200 mM Na_2HPO_4 (pH 8.5), and phage were amplified in *Escherichia coli* (DH5αF′). Affinity selection was repeated three times, and individual phage were isolated from either the second or third round of amplification. Peptide sequences were then deduced by DNA sequencing.
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9. TRF assays were performed at room temperature as follows: Costar (Cambridge, MA) high-binding 384-well plates were coated with streptavidin in 0.1 M sodium bicarbonate and blocked with BSA. Twenty microliters of biotinylated ERE (100 nM in TBST) was added to each well. After a 1-hour incubation, biotin (50 μM in TBST) was added to block any remaining binding sites. The plates were washed, and 20 μl of ERα (100 nM in TBST) was added to each well. After a 1-hour incubation, the plates were washed, and 5 μl of 5 μM 4-OH tamoxifen was added to each well followed by 15 μl of solution containing the peptides conjugated to unlabeled streptavidin (prepared as described below) at a range of concentrations (from 1.67 μM in twofold dilutions). After a 30-min incubation with the 4-OH tamoxifen and conjugate, 5 μl of 400 nM europium-labeled streptavidin (Wallac, Gaithersburg, MD)–biotinylated peptide conjugate (prepared as described below) was added and incubated for 1 hour. The plates were then washed, and the europium enhancement solution was added. Fluorescent readings were obtained with a POLARstar fluorimeter (BMG Lab Technologies, Durham, NC) with a <400-nm excitation filter and a 620-nm emission filter. The streptavidin-biotinylated peptide conjugates were prepared by adding 4 pmol of biotinylated peptide per picomole of streptavidin. After incubation on ice for 30 min, the remaining biotin-binding sites were blocked with biotin before addition to the ER-coated plate.
10. HepG2 cells were maintained in modified Eagle's medium (Life Technologies, Grand Island, NY) supplemented with 10% fetal bovine serum (Life Technologies). Transfections were performed as described [J. D. Norris *et al.*, *J. Biol. Chem.* **270**, 22777 (1995)]. pCMV–β-Gal and 5× GAL4-tata-Luc were described previously [B. L. Wagner, J. D. Norris, T. A. Knotts, N. L. Weigel, D. P. McDonnell, *Mol. Cell. Biol.* **18**, 1369 (1998)]. Gal4 DBD-peptide fusions were created as follows: Peptide-coding sequences were excised from mBAX vector with Xho I–Xba I and subcloned into pM vector (Clontech, Palo Alto, CA) with a linker sequence to generate Sal I and Xba I sites for cloning. ERα-VP16 was generated by polymerase chain reaction (PCR) of human ERα-cDNA containing Eco RI sites flanking both 5′ and 3′ termini. The resultant PCR product was then subcloned into pVP16 (Clontech). All PCR products were sequenced to ensure the fidelity of the resultant construct. 17β-estradiol, 4-hydroxy-tamoxifen, and nafoxidine were purchased from Sigma.
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15. ERβ-VP16 was generated by PCR of ERβ cDNA, and the resultant product was cloned into pVP16. Dexamethasone and progesterone were purchased from Sigma.
16. ERα expression vector pRST7-hER is reported elsewhere [S. L. Dana, P. A. Hoener, D. L. Wheeler, C. L. Lawrence, D. P. McDonnell, *Mol. Endocrinol.* **8**, 1193 (1994)].

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Rsp5 Ubiquitin-Protein Ligase Mediates DNA Damage-Induced Degradation of the Large Subunit of RNA Polymerase II in *Saccharomyces cerevisiae*

SYLVIE L. BEAUDENON,[1] MARIA R. HUACANI,[2] GUANGLI WANG,[1] DONALD P. MCDONNELL,[2] AND JON M. HUIBREGTSE[1]*

Department of Molecular Biology and Biochemistry, Rutgers University, Piscataway, New Jersey 08855,[1] and Department of Pharmacology and Cancer Biology, Duke University Medical Center, Durham, North Carolina 27710[2]

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Rsp5 is an E3 ubiquitin-protein ligase of *Saccharomyces cerevisiae* that belongs to the hect domain family of E3 proteins. We have previously shown that Rsp5 binds and ubiquitinates the largest subunit of RNA polymerase II, Rpb1, in vitro. We show here that Rpb1 ubiquitination and degradation are induced in vivo by UV irradiation and by the UV-mimetic compound 4-nitroquinoline-1-oxide (4-NQO) and that a functional *RSP5* gene product is required for this effect. The 26S proteasome is also required; a mutation of *SEN3/RPN2* (*sen3-1*), which encodes an essential regulatory subunit of the 26S proteasome, partially blocks 4-NQO-induced degradation of Rpb1. These results suggest that Rsp5-mediated ubiquitination and degradation of Rpb1 are components of the response to DNA damage. A human WW domain-containing hect (WW-hect) E3 protein closely related to Rsp5, Rpf1/hNedd4, also binds and ubiquitinates both yeast and human Rpb1 in vitro, suggesting that Rpf1 and/or another WW-hect E3 protein mediates UV-induced degradation of the large subunit of polymerase II in human cells.

Ubiquitin-dependent proteolysis involves the covalent ligation of ubiquitin to substrate proteins, which are then recognized and degraded by the 26S proteasome. While many of the components involved in catalyzing protein ubiquitination have been identified and characterized biochemically, we are only beginning to understand how the system specifically recognizes appropriate substrates. At least three classes of activities, known as E1 (ubiquitin-activating), E2 (ubiquitin-conjugating), and E3 (ubiquitin-protein ligase) enzymes, cooperate in catalyzing protein ubiquitination (34). The enzymatic mechanisms and functions of the E1 and E2 proteins have been well characterized. In contrast, the E3 enzymes are a diverse and less-well-characterized group of activities, and many lines of evidence indicate that E3 activities play a major role in determining the substrate specificity of the ubiquitination pathway (14, 28, 34).

The hect (homologous to E6-AP carboxyl terminus) domain defines a family of E3 proteins that were discovered through the characterization of human E6-AP (17). The interaction of E6-AP with the E6 protein of the cervical cancer-associated human papillomavirus types causes E6-AP to associate with and ubiquitinate p53, suggesting that E6 functions in promoting cellular immortalization by, at least in part, stimulating the destruction of this important tumor suppressor protein (16). The hect E3 molecular masses range from 92 to over 500 kDa, with the hect domain comprising the approximately 350 carboxyl-terminal amino acids (17, 34). Exactly five hect E3s are encoded by the *Saccharomyces cerevisiae* genome, and over 30 have been identified so far in mammalian species. An obligatory intermediate in the ubiquitination reactions catalyzed by hect E3s is a ubiquitin-thioester formed between the thiol group of an absolutely conserved cysteine within the hect domain and the terminal carboxyl group of ubiquitin (33). E3 becomes "charged" with ubiquitin via a cascade of ubiquitin-thioester transfers, in which ubiquitin is transferred from the active-site cysteine of an E1 enzyme to the active-site cysteine of an E2 enzyme and finally to hect E3, which catalyzes isopeptide bond formation between ubiquitin and the substrate. E3 can apparently be recharged with ubiquitin while bound to the substrate and can therefore catalyze ligation of multiple ubiquitin moieties to the substrate, through conjugation either to other lysines on the substrate or to lysine residues on previously conjugated ubiquitin molecules. The resulting multiubiquitinated substrate is then recognized and degraded by the 26S proteasome. Structure-function analyses of human E6-AP and yeast Rsp5 have suggested a model for hect E3 function in which the large and nonconserved amino-terminal domains of these proteins contain determinants for substrate specificity, while the carboxyl-terminal hect domain catalyzes the multiubiquitination of bound substrates (16, 39).

The *S. cerevisiae RSP5* gene encodes an essential hect E3 protein, and mutations in the gene have been isolated in multiple genetic screenings, including one for a suppressor of mutations in *SPT3* (reference 41; also cited in references 17 and 18). Spt3 is part of the TATA-binding protein recognition component of the SAGA complex, which plays an important role in transcriptional activation in vivo and contains histone acetyltransferase activity (37). Rsp5 has also been identified as being involved in the down-regulation of several plasma membrane-associated permeases, including uracil permease (Fur4), general amino acid permease (Gap1), maltose permease (Mal61), and the plasma membrane H^+-ATPase (5, 9, 13, 23). The primary structure of yeast Rsp5 reveals, in addition to its carboxyl-terminal hect domain, two types of domains within the amino-terminal region: C2 (one domain between amino acids 3 and 140) and WW (three domains between amino acids 231 and 418). C2 domains interact with membrane phospho-

* Corresponding author. Mailing address: Department of Molecular Biology and Biochemistry, Rutgers University, Picataway, NJ 08855. Phone: (732) 445-0938. Fax: (732) 445-4213. E-mail: huibregt@waksman.rutgers.edu.

lipids, inositol polyphosphates, and proteins, in most cases dependent on or regulated by Ca^{2+} (31). Although it has not yet been demonstrated, it is possible that the C2 domain of Rsp5 is involved in targeting its membrane-associated substrates either by localizing Rsp5 to the plasma membrane or by directly mediating the interactions with these substrates.

WW domains are protein-protein interaction modules that recognize proline-rich sequences, with the consensus binding site containing either a PPXY (4, 21), PPLP (1a, 7), or PPPGM (2) sequence. WW domains, like SH3 domains, recognize polyproline ligands with high specificity but low affinity (K_d = 1 to 200 μM). The basis of recognition is the N-substituted nature of the proline peptide backbone rather than the proline side chain itself (26). It has been suggested that this explains how WW and SH3 domains can achieve specific but low-affinity recognition of ligands, since proline is the only natural N-substituted amino acid. It has also recently been shown that WW domains can recognize phosphoserine- and phosphothreonine-containing ligands (22), which has important implications for the diversity of substrates that may be recognized by Rsp5 and other WW domain-containing hect E3s. A structure-function analysis of Rsp5 showed that the hect domain and the region spanning WW domains 2 and 3 are necessary and sufficient to support the essential in vivo function of Rsp5, while the C2 domain and WW domain 1 are dispensable, at least under standard growth conditions (39). Together, the results of our structure-function analyses imply that ubiquitination of one or more substrates of Rsp5 is essential for cell viability and that the critical substrate(s) is recognized by the region containing WW domains 2 and 3.

Members of our group previously reported the results of a biochemical approach for identifying substrates of Rsp5, which led to the identification of Rpb1, the largest subunit (LS) of RNA polymerase II (Pol II), as a substrate of Rsp5 (18). Rpb1 is very efficiently ubiquitinated by Rsp5 in vitro, and the WW domain region mediates binding to Rpb1, with WW domain 2 being most critical. Since the requirements for Rpb1 binding and ubiquitination parallel those for the essential function of Rsp5, Rpb1 is a candidate for being at least one of the substrates related to the essential function of Rsp5. The biological relevance of Rpb1 ubiquitination was not initially clear, however, since Rpb1 is an abundant, long-lived protein in vivo. Interestingly, another study showed that the Pol II LS is subject to ubiquitination and degradation in response to UV irradiation (3, 30); however, the enzymatic components of the ubiquitin system responsible for this phenomenon were not identified or characterized. We show here that UV irradiation or treatment with a UV-mimetic chemical induces the degradation of Rpb1 in yeast cells and that Rsp5 and the 26S proteasome mediate this effect. Furthermore, we show that human Rpf1, a WW domain-containing hect (WW-hect) E3 protein, binds and ubiquitinates Rpb1 in vitro, suggesting that this may be the E3 protein that mediates UV-induced degradation of the Pol II LS in human cells.

MATERIALS AND METHODS

Yeast strains and plasmids. FY56 (*RSP5*), FW1808 (*rsp5-1*), and the *Gal-RSP5* strain were described previously (18, 39). The *sen3-1* (MHY811) and *SEN3* (MHY810) strains (6) were kindly provided by Mark Hochstrasser (University of Chicago). The *tom1* null mutant strain was made by single-step gene disruption in the diploid strain W303, and haploid *tom1*Δ colonies were isolated by the sporulation and dissection of the heterozygous *TOM1/tom1*Δ diploid. All plasmids that promote the expression of Rsp5 and Rpb1 were described previously (18, 39). Plasmids that promote the bacterial expression of glutathione *S*-transferase (GST)–Rpf1 fusion proteins were generated by PCR amplification of regions of the Rpf1 open reading frame in plasmid pBKC-hRPF1 (19). The GST-Rpf1 N protein contains amino acids 13 to 192 of Rpf1, the GST-WW protein contains amino acids 193 to 506, the GST-C protein contains amino acids 506 to 901, and the GST–WW-hect protein contains amino acids 193 to 901. This numbering is based on the assumption that amino acid 29 of the protein sequence given in GenBank (accession no. D42055) is the initiating methionine. pGEX-5x-1 (Pharmacia, Piscataway, N.J.) was the cloning vector for the expression of all the GST fusion proteins except for GST–WW-hect, which was expressed by pGEX-6p-1.

Protein purification and biochemical assays. GST fusion proteins for ubiquitination assays and protein binding assays were expressed in *Escherichia coli* by standard methods and affinity purified on glutathione-Sepharose (Pharmacia). Ubiquitination assays utilized hect E3 proteins (Rsp5, the Rsp5 C-A mutant, human E6-AP, and Rpf1 WW-hect) that were cleaved from the GST portion of the molecule with PreScission protease (Pharmacia). These proteins were then used in ubiquitination assays with ^{35}S-labeled yeast Rpb1 that had been translated in vitro with a TNT rabbit reticulocyte lysate system (Promega, Madison, Wis.), as described previously (18).

Rpb1 binding assays were performed by mixing 100 ng of GST-E3 fusion protein bound to 10 μl of glutathione-Sepharose with 80 μg of total HeLa cell lysate (cell lysis buffer: 0.1 M Tris [pH 8.0], 0.1 M NaCl, and 1% NP-40), with the remainder of the 125-μl volume consisting of 25 mM Tris (pH 8.0) and 125 mM NaCl. Reaction mixtures were rotated for 2 h at 4°C, and the beads were washed three times with 500 μl of cell lysis buffer. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) loading buffer was added directly to the Sepharose and heated at 95°C for 5 min, and proteins were analyzed by SDS-PAGE and Western blotting with either anti-carboxyl-terminal domain (anti-CTD) antibody (generously provided by Danny Reinberg, University of Medicine and Dentistry of New Jersey, Piscataway) or anti-Pol II antibody N-20 from Santa Cruz Biotechnology (Santa Cruz, Calif.).

Analysis of UV- and 4-NQO-treated cells. HeLa cells were maintained in Dulbecco's modified Eagle medium with 10% fetal bovine serum, and UV irradiation was performed on tissue culture dishes after the removal of the medium. A germicidal lamp emitting light at 254 nm with an incident dose rate of 1.5 J per m^2 per s was used, and the time of irradiation was generally 15 s, for a total dose of 22.5 J per m^2. Fresh medium was then added to the cells, which were then allowed to recover for various times at 37°C. 4-Nitroquinoline-1-oxide (4-NQO) (Sigma), prepared as a 0.5-mg/ml stock solution in ethanol, was added directly to the medium at various concentrations and times. Extracts were made by lysing cells directly in SDS-PAGE loading buffer.

Yeast cells were irradiated as follows. Log-phase liquid cultures (5 optical density [OD] units) were concentrated by centrifugation to 0.5 ml, and then the cells were spread onto 10-cm agar plates. The liquid was allowed to absorb into the plates for 30 min at 30°C, and then the plates were irradiated for 15 s, as described above for HeLa cells. The cells were then collected from the plates and extracts were prepared as described below. Log-phase liquid yeast cultures (5 OD units) were treated with 4-NQO by adding a 0.5-mg/ml stock solution in ethanol directly to the culture medium for either 30 or 60 min. Yeast cell extracts were prepared by the method of Silver et al. (36). Briefly, 5 OD units of cells were resuspended in 1 ml of 0.25 M NaOH–1% β-mercaptoethanol and incubated on ice for 10 min. A volume of 0.16 ml of 50% trichloroacetic acid was added, and incubation on ice was continued for 10 min. The precipitate was collected by microcentrifugation at 4°C for 10 min, and then the pellet was washed with cold acetone, dried, and resuspended in 200 μl of SDS-PAGE sample buffer. Samples were heated at 95°C for 10 min prior to being loaded onto SDS-PAGE gels. Protein from the equivalent of 0.1 to 0.25 OD unit of cells was analyzed on SDS–7% PAGE gels for Western analyses of Rpb1. Immunoprecipitation and Western blotting (see Fig. 4) were performed by diluting 40 μl of yeast extract with 1.4 ml of 25 mM Tris (pH 7.9)–125 mM NaCl, followed by the addition of antibody and 20 μl of protein A-Sepharose (Pharmacia). The mixture was rotated at 4°C for 4 h; the Sepharose beads were collected, washed, and boiled in sample buffer; and then the proteins were analyzed by SDS-PAGE followed by immunoblotting.

Antibodies utilized in this study were either anti-CTD rabbit polyclonal antibody (used for yeast Rpb1 Western analyses and Rpb1 immunoprecipitations; generously provided by Danny Reinberg), anti-human Pol II rabbit polyclonal antibody N-20 (Santa Cruz Biotechnology), antiubiquitin mouse monoclonal antibody (Santa Cruz Biotechnology), antihemagglutinin rabbit polyclonal antibody (Santa Cruz Biotechnology), anti-Rsp5 mouse monoclonal antibody (39), or anti-Rfa1 rabbit polyclonal antibody (generously provided by Steve Brill, Rutgers University). Horseradish peroxidase-linked secondary antibodies and chemiluminescent reagents were obtained from DuPont NEN.

RESULTS

UV irradiation and 4-NQO induce the degradation of Rpb1 in both human and yeast cells. 4-NQO is considered a UV mimetic because it is metabolized to yield a compound that reacts with purine nucleotides of DNA, and these adducts are processed by the nucleotide excision repair (NER) system in a manner similar to that of dipyrimidine photoproducts induced by 254-nm UV light (15, 29). It was previously shown that UV irradiation of human cells induces the ubiquitination and deg-

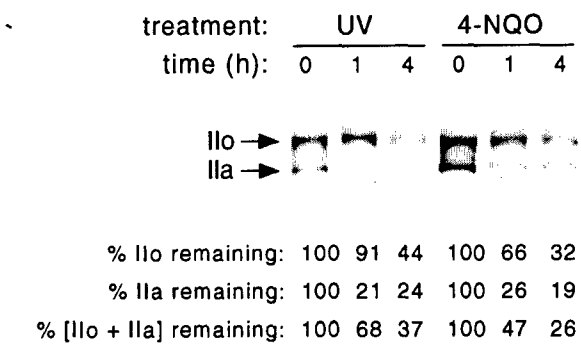

FIG. 1. hRpb1 levels following UV irradiation and 4-NQO treatment of HeLa cells. HeLa cells were irradiated with 254-nm UV light at 22.5 J per m^2 as described in Materials and Methods, and cell extracts were prepared immediately or 1 or 4 h postirradiation. For 4-NQO treatment, the chemical was added directly to the culture medium at a final concentration of 0.5 μg/ml, and cell extracts were prepared immediately or 1 or 4 h later. Relative hRpb1 levels were determined by SDS-PAGE and immunoblotting and quantitated by densitometry. Levels are expressed as the percentage of Rpb1 remaining relative to the level in untreated cells.

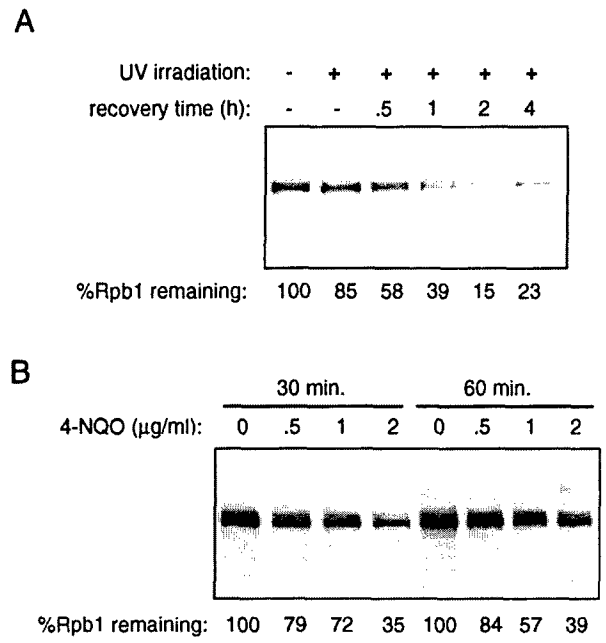

FIG. 2. (A) Rpb1 levels following UV irradiation of yeast. Yeast cells (strain FY56) were irradiated at 22.5 J per m^2 as described in Materials and Methods, and whole-cell extracts were made at the indicated times postirradiation. Rpb1 was detected by SDS-PAGE followed by immunoblotting with anti-CTD antibody. Rpb1 levels were quantitated by densitometry and are expressed as the percentage of Rpb1 remaining relative to the level in untreated cells. (B) Rpb1 levels following 4-NQO treatment. 4-NQO was added to liquid cultures of log-phase yeast at the indicated concentrations, and cells were collected at the indicated times following addition. Whole-cell extracts were prepared, and Rpb1 was detected by SDS-PAGE and immunoblotting.

radation of human Rpb1 (hRpb1) (3, 30). Figure 1 demonstrates this effect in HeLa cells. Cells were irradiated with 254-nm UV light at a dose of 22.5 J per m^2, and cell extracts were made at various times, up to 4 h after irradiation. Extracts were analyzed by SDS-PAGE, followed by immunoblotting with an antibody that recognizes the amino-terminal region of hRpb1 and therefore detects both hypophosphorylated (IIa) and hyperphosphorylated (IIo) forms of the protein. The degradation of the IIa form was more rapid and more complete than the degradation of the IIo form, with the IIa form reaching a minimum degradation of 20 to 25% of the initial amount after 1 h, while the IIa form reached a minimum degradation of 40 to 50% of the initial amount after 4 h. 4-NQO treatment stimulated the degradation of hRpb1 over a similar time course, again with the IIa form disappearing more rapidly and more completely than the IIo form. Lactacystin, a highly specific inhibitor of the proteasome, inhibited both UV- and 4-NQO-induced degradation of hRpb1 (not shown), which is consistent with previous reports that this effect is mediated by the 26S proteasome of the ubiquitin system (30).

Figure 2 shows that the degradation of Rpb1 was also induced in *S. cerevisiae* by both UV irradiation and 4-NQO treatment. UV irradiation of intact yeast cells on agar plates led to a dose- and time-dependent decrease in the steady-state level of Rpb1 (Fig. 2A). Rpb1 levels reached a minimum of 15 to 20% of the initial amount between 1 and 2 h after irradiation and began to return to normal after 4 h. 4-NQO also elicited a dose-dependent decrease in Rpb1 levels, reaching a minimum 30 to 60 min after the addition of 4-NQO (Fig. 2B). The amount of Rpb1 remaining in the experiment whose results are shown was 35 to 40% of the initial amount; however, in other experiments, the minimum was generally 25 to 30% (Fig. 3). Neither UV irradiation nor 4-NQO treatment resulted in a significant loss of viability at doses necessary to elicit maximal Rpb1 degradation. Unlike hRpb1, the hypo- and hyperphosphorylated forms of yeast Rpb1 migrate as a very closely spaced doublet and are not easily distinguished by SDS-PAGE. Therefore, it is difficult to conclude whether there is an apparent preferential disappearance of one form over the other, as there is with hRpb1.

To rule out the possibility that the decrease in yeast Rpb1 levels accompanying UV or 4-NQO treatment was simply the result of inhibition of the synthesis of Rpb1, 4-NQO-treated cells were compared to cells treated with cycloheximide. As shown in Fig. 3A, cycloheximide treatment led to only a slight decrease in Rpb1 levels after 45 min, whereas 4-NQO treatment resulted in the reduction in Rpb1 levels as described above. Total cellular protein levels were not affected by 4-NQO treatment, and Coomassie blue staining of SDS-PAGE gels indicated that the effect of 4-NQO was specific for Rpb1. This was confirmed by immunoblotting for an unrelated nuclear protein, Rfa1, a component of replication protein A. Figure 3B shows that levels of Rfa1 were not affected by 4-NQO treatment under conditions in which Rpb1 degradation was induced.

The appearance of slower-migrating forms of Rpb1, suggestive of ubiquitinated intermediates, was evident in some experiments at higher concentrations of 4-NQO and on longer film exposures. These slower-migrating bands were shown to be ubiquitinated forms of Rpb1 by immunoprecipitating them with anti-Rpb1 antibody, followed by immunoblotting with either anti-CTD or antiubiquitin antibody (Fig. 4). While the accumulation of ubiquitinated forms of Rpb1 was clearly stimulated by 4-NQO, there was some reaction of the Rpb1 immunoprecipitate with the antiubiquitin antibody even in untreated cells. This may reflect a basal level of Rpb1 ubiquitination in normally growing cells, as suggested previously (18).

4-NQO-induced degradation of Rpb1 is dependent on *RSP5* and *SEN3/RPN2*. Members of our group previously showed that Rsp5 ubiquitinates Rpb1 in vitro (18). To determine if the in vivo-induced degradation of Rpb1 was dependent on Rsp5, we first took advantage of a yeast strain that contains a single copy of a conditionally expressed wild-type *RSP5* gene. The

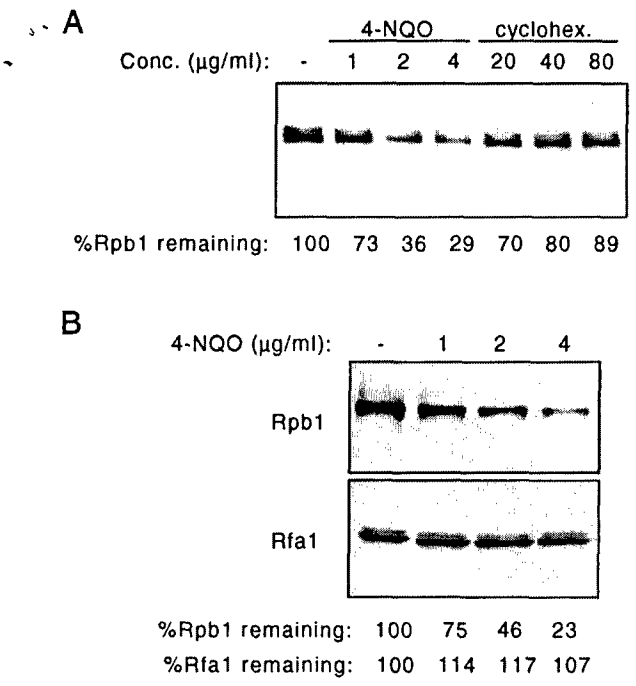

FIG. 3. (A) Yeast cells (FY56 [*RSP5*]) were treated with the indicated doses of 4-NQO or cycloheximide (cyclohex.) for 30 min, cell extracts were prepared, and Rpb1 levels were examined by SDS-PAGE and immunoblotting with anti-CTD antibody. (B) Yeast cells (FY56 [*RSP5*]) were treated with the indicated doses of 4-NQO for 30 min, cell extracts were prepared, and Rpb1 levels and Rfa1 levels were examined by SDS-PAGE and immunoblotting.

Gal-RSP5 yeast strain contains an epitope-tagged *RSP5* gene under the control of the *GAL1* promoter, which is integrated at the *RSP5* chromosomal locus (18). This strain was grown to early log phase in galactose-containing medium, and then it was switched to dextrose-containing medium for 48 h. Figure 5A shows that Rsp5 protein levels were dramatically reduced after 48 h in dextrose. The cells were still fully viable at this point and resumed growth when shifted back to galactose-containing medium. The dextrose-shifted cells were treated with 4-NQO and compared to log-phase cells that had been maintained in galactose-containing medium. 4-NQO-induced Rpb1 degradation occurred in the cells maintained in galactose, but not in Rsp5-depleted cells. These results suggest that 4-NQO-induced degradation of Rpb1 is dependent on *RSP5*.

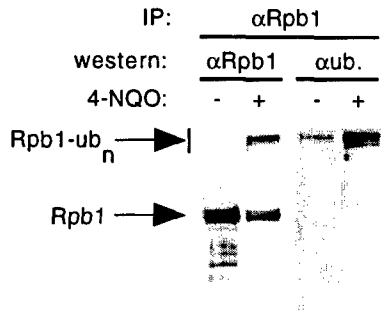

FIG. 4. Antiubiquitin antibody recognizes high-molecular-weight forms of Rpb1 from 4-NQO-treated cells. Yeast cells were treated with 4-NQO at 4 μg/ml for 30 min, and whole-cell extracts were prepared. Rpb1 was immunoprecipitated (IP) in duplicate from each sample with anti-CTD antibody. The immunoprecipitates were then analyzed by SDS-PAGE followed by immunoblotting with either anti-CTD (αRpb1) or antiubiquitin (αub.) antibody.

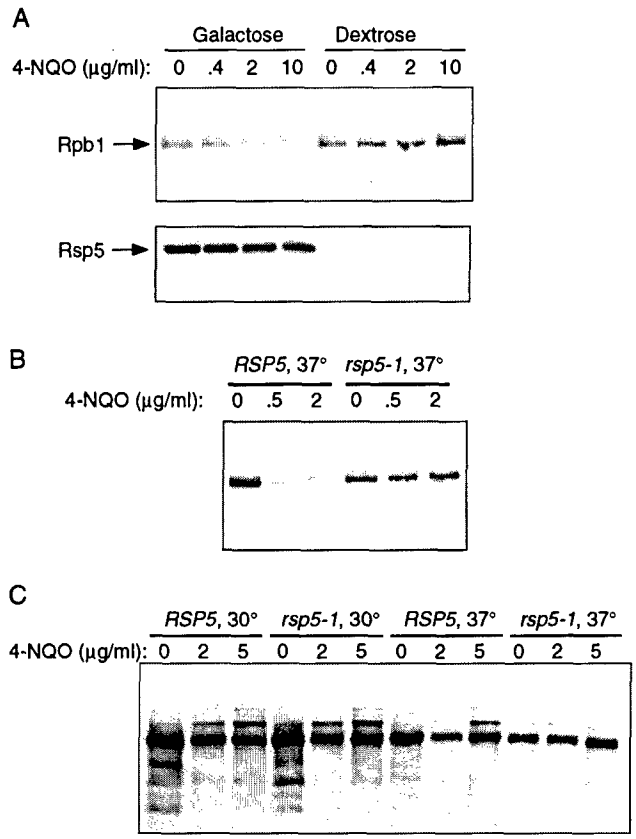

FIG. 5. (A) 4-NQO treatment of the *Gal-RSP5* strain maintained in galactose or shifted to dextrose. The *Gal-RSP5* strain was grown to early log phase in galactose-containing medium, and then the cells were either shifted to dextrose-containing medium for 48 h or maintained in galactose-containing medium. The cultures were then treated with 4-NQO at the indicated concentrations for 30 min, and whole-cell extracts were prepared and analyzed by SDS-PAGE and immunoblotting with either an anti-Rsp5 monoclonal antibody (bottom) or anti-CTD antibody (top). (B) 4-NQO treatment of the *rsp5-1* temperature-sensitive mutant. Strains FY56 (*RSP5*) and FW1808 (*rsp5-1*) were grown to mid-log phase at 30°C and then shifted to 37°C for 1 h. 4-NQO was then added at the indicated concentrations for 30 min. Whole-cell extracts were prepared, and Rpb1 was detected by SDS-PAGE and immunoblotting. (C) Experiment similar to that in panel B, except that cells were treated with 4-NQO at both 30 and 37°C.

To independently confirm the importance of Rsp5 in the induced degradation of Rpb1, we examined the effect of 4-NQO on the temperature-sensitive *rsp5-1* mutant. Temperature sensitivity is conferred by a single amino acid change (amino acid 733) within the hect domain that directly affects the catalytic activity of the protein (39). The *rsp5-1* strain grows with a slightly longer doubling time than an isogenic *RSP5* strain at 30°C but arrests within 30 to 60 min after a shift to 37°C. Figure 5B shows that Rpb1 degradation was induced by 4-NQO in an isogenic wild-type *RSP5* strain at 37°C, while little or no loss of Rpb1 was seen in the *rsp5-1* strain at 37°C. Figure 5C shows the results of an experiment in which multiubiquitinated forms of Rpb1 were evident following 4-NQO treatment. The accumulation of these forms was seen in the wild-type *RSP5* strain at both 30 and 37°C and in the *rsp5-1* strain at 30°C, but not at 37°C. These results again indicate that 4-NQO-induced ubiquitination and degradation of Rpb1 are *RSP5* dependent.

A strain containing a mutation in a subunit of the 26S pro-

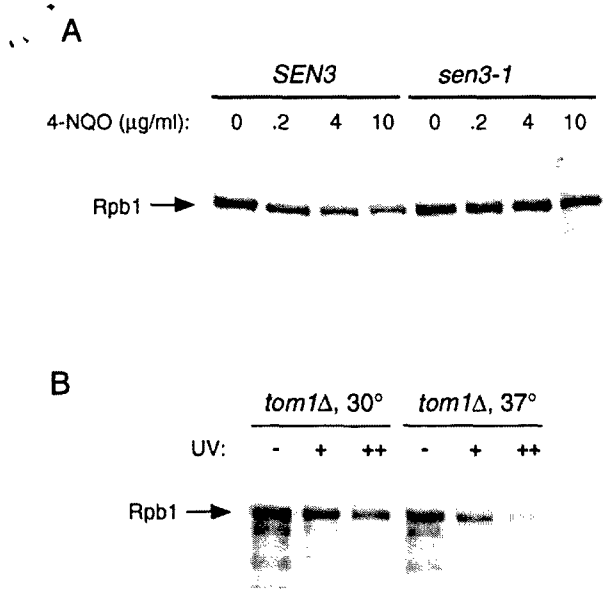

FIG. 6. (A) 4-NQO treatment of *SEN3* and *sen3-1* strains at 37°C. 4-NQO was added at the indicated concentrations for 30 min. Whole-cell extracts were prepared, and Rpb1 was detected by SDS-PAGE and immunoblotting. (B) The *tom1*Δ mutant was grown at 30°C and then either maintained at 30°C or shifted for 4 h to 37°C. Cells were then irradiated at either 25 (+) or 50 (++) J/m^2, followed by a 1-h recovery period at their respective temperatures. Whole-cell extracts were then prepared, and Rpb1 was detected by SDS-PAGE and immunoblotting.

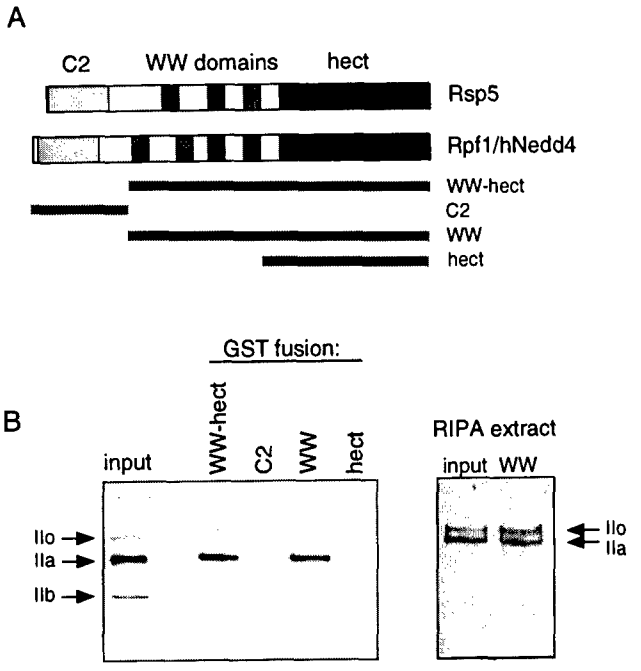

FIG. 7. (A) Schematic representation of yeast Rsp5 and human Rpf1/Nedd4. GST-Rpf1 fusions to the regions of Rpf1 indicated by the solid bars were made. (B) (Left) HeLa cell extract was prepared in NP-40 lysis buffer (see Materials and Methods). The binding of hRpb1 to GST-Rpf1 fusion proteins immobilized on glutathione-Sepharose was analyzed by SDS-PAGE and immunoblotting. The "input" shows hRpb1 in the extract with forms IIo, IIa, and IIb. (Right) Similar experiment, with HeLa cell extract prepared in radioimmunoprecipitation assay (RIPA) buffer. The input and binding to GST-WW are shown.

teasome was used to determine if the 4-NQO-induced degradation of Rpb1 was proteasome dependent. *SEN3/RPN2* encodes an essential non-ATPase regulatory subunit of the 26S proteasome (6). The *sen3-1* mutant shows a growth defect at 30°C (doubling time of 4.5 h) and a more severe growth defect at higher temperatures. The MATα2 transcription factor and certain artificial substrates of the ubiquitin system (Ub-Pro-β-galactosidase and Ub-Leu-β-galactosidase) have been shown to be stabilized in this mutant at 30°C. We compared the *sen3-1* mutant to an isogenic wild-type *SEN3* strain for its ability to support 4-NQO-induced degradation of Rpb1. As shown in Fig. 6A, the *sen3-1* mutant was defective in 4-NQO-induced degradation of Rpb1 compared to the *SEN3* strain. This result indicates that UV-induced degradation of Rpb1 is proteasome dependent, consistent with the observation that proteasome inhibitors blocked the degradation of the human Pol II LS in response to UV irradiation (30).

A caveat to the experiments utilizing the *GAL-RSP5*, *rsp5-1*, and *sen3-1* strains is that both *RSP5* and *SEN3/RPN2* are essential genes, and their inactivation results in growth inhibition. Therefore, indirect effects cannot be ruled out as being responsible for the block in 4-NQO-induced Rpb1 degradation seen in these mutants. To rule out the possibility that the block in Rpb1 degradation is due to a general growth arrest, we examined a temperature-sensitive mutation in a gene not predicted to affect either Rsp5 or Rpb1. We examined a *tom1* null mutant, since *TOM1* encodes a hect E3 protein that does not interact with Rpb1. Interestingly, Tom1 appears to influence transcription through effects on ADA coactivators, possibly by targeting the Spt7 protein for ubiquitination (32). The *tom1* null mutant has a near-normal doubling time at 30°C but exhibits a strong growth arrest within 2 h after a shift to 37°C. The *tom1* mutant was UV irradiated either at 30°C or 4 h after a shift to 37°C, and Rpb1 levels were examined. Figure 6B shows that the degradation of Rpb1 was induced at both temperatures. Therefore, the lack of induced degradation in the *rsp5* and *sen3* mutants is unlikely to be due to general growth arrest or cell stress.

Rpf1/hNedd4, a human hect E3 protein related to Rsp5, binds and ubiquitinates Rpb1 in vitro. Rpf1, also known as human Nedd4 (hNedd4), has a C2 domain at its extreme amino terminus, four WW domains in the central portion of the molecule, and a carboxyl-terminal hect domain (Fig. 7A). Rpf1 is one of at least seven human hect E3s that have this general organization, with a variable number of WW domains (two to four). GST-Rpf1 proteins were expressed as indicated in Fig. 7A, and equivalents amounts (100 ng) of each protein were assayed for the ability to bind to hRpb1. The full-length Rpf1 protein was not used in this analysis because it was produced in small amounts in bacteria and, furthermore, was not catalytically active, as judged by ubiquitin-thioester assays (not shown). Rpf1 WW-hect and the isolated WW domain region stably bound the hRpb1 present in the HeLa cell extract (Fig. 7B, left panel), whereas neither the isolated C2 domain nor the hect domain bound to hRpb1. These results are consistent with previous results showing that the WW domain region of Rsp5 is necessary and sufficient for binding to yeast Rpb1 (18, 39). In addition, a well-characterized proteolyzed form of hRpb1 (form IIb) that lacks the CTD did not bind to Rpf1, also consistent with previous results showing that the CTD is the binding site for Rsp5 (18, 39). There was an apparent preferential binding of Rpf1 to the hypophosphorylated (IIa) form of hRpb1 in this experiment; however, the degree to which the phosphorylated (IIo) form of hRpb1 associated with Rpf1 was dependent on the cell extraction buffer. When the cell lysis buffer conditions were harsher (radioimmunoprecipitation assay buffer instead of NP-40 lysis buffer [11]), an equiv-

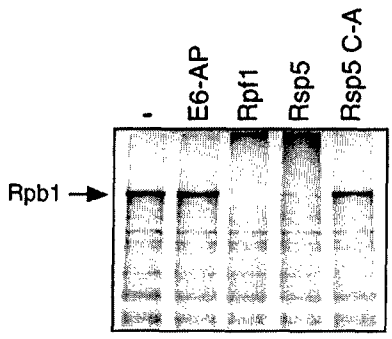

FIG. 8. Ubiquitination of Rpb1 by Rpf1 in vitro. Rpb1 was translated in vitro in rabbit reticulocyte lysate in the presence of [^{35}S]-methionine. Purified hect E3 proteins (human E6-AP, human Rpf1 [WW-hect; amino acids 193 to 901], yeast Rsp5, and the mutant of Rsp5 with a change of the active-site Cys to Ala [C-A]) were incubated as indicated with Rpb1 in the presence of ATP, ubiquitin, E1 enzyme, and E2 enzyme (*Arabidopsis thaliana* Ubc8) as previously described (18).

alent portion of hyperphosphorylated hRpb1 bound to Rpf1 (Fig. 7B, right panel). This suggests that the interaction of the hyperphosphorylated CTD with other proteins might preclude binding to Rpf1 and that Rsp5 and Rpf1 have an inherent ability to bind to both forms of the protein. This interpretation is consistent with previous results showing that Rsp5 could bind to both the IIo and IIa forms of purified Pol II holoenzyme in vitro (18).

To determine if Rpf1 can ubiquitinate Rpb1, the Rpf1 WW-hect protein was cleaved from the purified GST fusion protein and assayed for its ability to ubiquitinate in vitro-translated yeast Rpb1. Rpf1 was as efficient in stimulating multiubiquitination of Rpb1 as yeast Rsp5 (Fig. 8). Neither the mutant of Rsp5 with a change of the active-site cysteine to alanine nor human E6-AP ubiquitinated Rpb1. Together, the binding and ubiquitination results suggest that Rpf1 may mediate the DNA damage-induced degradation of the Pol II LS in human cells.

DISCUSSION

Rpb1 was initially identified as a substrate of Rsp5 based on a biochemical screening for proteins that were bound and ubiquitinated by Rsp5 in vitro (18). While Rsp5 was found to efficiently multiubiquitinate Rpb1 in vitro, the biological function of this was unclear, since Rpb1 is an abundant and stable protein in vivo. The steady-state level of Rpb1 was found to increase modestly (approximately three- to fivefold) on prolonged transcriptional repression of *RSP5*, providing evidence that Rpb1 may be a bona fide substrate of Rsp5 in vivo, even if the half-life of Rpb1 under normal growth conditions is relatively long. Other studies have shown that the inhibition of transcription caused by the exposure of mammalian cells to DNA-damaging agents or treatment, including α-amanitin, actinomycin D, cisplatin, and UV irradiation, leads to the degradation of the Pol II LS (3, 27). Ratner et al. further demonstrated that the degradation of the Pol II LS induced by UV irradiation was ubiquitin and proteasome dependent (30). Together, these results suggested that the recognition of Rpb1 by Rsp5 might be enhanced in response to DNA damage. The experiments described here showed that, as in human cells, DNA damage induces the ubiquitination and degradation of Rpb1 in *S. cerevisiae* and that this is dependent on the Rsp5 ubiquitin-protein ligase. In addition, a human hect E3 protein closely related to Rsp5, Rpf1/hNedd4, is shown to bind and ubiquitinate Rpb1 in vitro, suggesting that this hect E3 protein might mediate UV-induced degradation of Rpb1 in human cells.

It has long been recognized that RNA synthesis is down-regulated in response to DNA damage and that stalled RNA polymerase at sites of DNA damage might serve as a signal for the recruitment of the NER machinery (10, 24). This is thought to be the basis of a specialized form of NER, transcription-coupled repair (TCR), in which lesions within the transcribed strand of genes are repaired more rapidly than lesions on the nontranscribed strand or outside of the transcription units. TCR also occurs in *E. coli*, where the transcription repair coupling factor binds to and releases RNA polymerase stalled at a lesion and then stimulates the recruitment of the repair machinery (35). Several lines of evidence suggest that the mechanism of TCR is more complex in eukaryotes, and it is generally thought that a stalled RNA polymerase can resume transcript synthesis following repair. This is based in part on the stability of stalled RNA polymerase-template-RNA complexes in vitro and the idea that it would be energetically wasteful to abort transcript synthesis entirely. The finding that a fraction of the Pol II LS is ubiquitinated and degraded in response to DNA damage suggests an alternative mechanism for the down-regulation of transcription in response to DNA damage: irreversible disassembly of transcription complexes by the degradation of the major catalytic subunit of Pol II.

It is not yet clear which form of Pol II is targeted for ubiquitin-mediated degradation following DNA damage. The CTD, which is necessary and sufficient for Rsp5 binding, is subject to phosphorylation and dephosphorylation events during the transcription cycle and is also the site of interaction of many components of the transcription machinery (25). The CTD is hypophosphorylated (IIa) in Pol II transcription initiation complexes and undergoes phosphorylation upon promoter clearance to yield a hyperphosphorylated (IIo) form that persists throughout transcription elongation. Ratner et al. (30) reported that ubiquitinated forms of hRpb1 detected after UV irradiation reacted with an antibody that is specific for the hyperphosphorylated form of hRpb1, suggesting that Pol II complexes arrested at intragenic damage sites might be the preferential substrate for ubiquitination. This is not consistent, however, with the observation that the hypophosphorylated form of hRpb1 preferentially disappears in response to either UV irradiation or 4-NQO treatment. In order to explain this discrepancy, Ratner et al. suggested that the apparent loss of hypophosphorylated hRpb1 upon UV irradiation might reflect a rapid conversion of hypo- to hyperphosphorylated Rpb1 in order to compensate for the loss of hyperphosphorylated Rpb1. While we cannot exclude this possibility, the data are also consistent with a model in which the hypophosphorylated form of Pol II is actually the preferential substrate for ubiquitination but that the kinetics of its ubiquitination and degradation are too rapid to allow the detection of ubiquitinated intermediates.

While further studies are clearly necessary to determine which form of Pol II is targeted for ubiquitin-mediated degradation in response to DNA damage in vivo, our in vitro results suggest that there is not a specific requirement for the recognition of Rpb1 by Rsp5 in terms of the phosphorylation state of the CTD. Phosphorylation of the CTD is not a prerequisite for Rsp5 recognition, since in vitro-translated Rpb1 and GST-CTD produced in bacteria are both efficiently recognized by Rsp5. We also showed previously that the hypo- and hyperphosphorylated forms of purified human Pol II holoenzyme bind equally well to GST-Rsp5 (18). In addition, both Rsp5 and Rpf1 bind to the hypophosphorylated form of hRpb1 present in human cell extracts; however, the degree to which

Rsp5 and Rpf1 can bind to hyperphosphorylated hRpb1 is a function of the cell extraction buffer, with more stringent extraction buffers resulting in more binding of the hyperphosphorylated forms. Together, these results suggest that the association of other transcription factors with Pol II, and specifically with the CTD, might block recognition by Rsp5 in vivo. Changes in Pol II transcription complexes in response to DNA damage, such as the dissociation of specific CTD-associated proteins or the dissociation of the elongated polymerase complex from the template, might then allow Rsp5 to bind and ubiquitinate Rpb1.

Rsp5 is the only hect E3 protein in yeast that has a C2 domain and WW domains, while at least seven human hect E3s with C2 and WW domains have been identified. The WW domains, as well characterized protein-protein interaction modules, are likely to mediate the interaction with at least some of the substrates of Rsp5, including Rpb1 (39). WW domains bind proline-rich ligands, with the best-characterized ligand being the PY motif (containing a PPXY sequence). In addition, it has recently been shown that WW domains can also recognize phosphoserine- and phosphothreonine-containing ligands (22), suggesting that there are two disparate types of WW domain ligands. The CTD heptapeptide consensus (YSPTSPS) may be a nonconsensus PY motif in the context of the repeating heptapeptide (YXPXXPXYXPXXPX). Alternatively, if the phosphorylated form of Rpb1 is the in vivo substrate of Rsp5, phosphorylation at the serine and/or threonine residues may contribute to recognition, although as mentioned above, phosphorylation is not required for the binding of Rsp5 to the CTD in vitro. Our finding that Rpf1/hNedd4 can bind and ubiquitinate hRpb1 in vitro suggests that this may be the E3 enzyme responsible for this effect in human cells. Preliminary results, however, indicate that other WW-hect E3s can also bind to Rpb1 in vitro (1). It is possible that while several of the WW-hect E3s can bind and ubiquitinate Rpb1 in vitro, intracellular localization is the key determinant of which E3 can target Rpb1 in vivo. Mouse Nedd4 and yeast Rsp5 are primarily cytoplasmic (12, 40); however, there is now a precedent for the ubiquitin-mediated degradation of nuclear proteins being linked to their export from the nucleus to the cytoplasm (8, 38).

While it is now established that DNA damage induces the degradation of Rpb1 in both yeast and human cells, the relevance of this to DNA repair is not yet clear. Rsp5 mutants do not show any apparent UV sensitivity, although we cannot yet rule out more subtle effects of Rsp5 on the efficiency of DNA repair. The fact that both CSA and CSB Cockayne syndrome cells were found to be defective in UV-induced Rpb1 degradation in human cells suggested that this is related to the process of TCR. However, a *rad26* null mutant (Rad26 is the yeast CSB homolog and the only yeast protein known to be required for TCR but not for NER) exhibited no defect in 4-NQO-induced Rpb1 degradation (data not shown). This suggests that TCR may not be directly linked to DNA damage-induced degradation of Rpb1, at least in yeast, and again raises the question of which form of Pol II is the in vivo substrate of Rsp5. The expression of Rpf1/hNedd4 in yeast cannot functionally substitute for expression by *RSP5* in terms of either cell viability or the UV-induced effect on Rpb1 (data not shown). The basis of this noncomplementation is not known but could be related to an inability of Rpf1 to productively interact with other components of the ubiquitin system in yeast.

Several examples of regulated substrate ubiquitination have now been characterized. In many cases, modification of the substrate, often by phosphorylation, can serve as a signal for recognition by specific E3 ubiquitin-protein ligases, as in the recognition of phosphorylated Sic1 by SCF^{Cdc4} (28). In other cases, the unmasking of ubiquitination signals can occur when a substrate dissociates from an interacting protein, as in the case of the mutual destruction of the MATα2 and MATa1 transcription factors upon the dissociation of the heterodimer (20). An unmasking of the recognition signals on Rpb1 in response to DNA damage may account for the observations that Rpb1 is freely and efficiently recognized by Rsp5 under several different experimental conditions in vitro yet is normally a stable and long-lived protein in vivo. It seems likely that the nature of the Rpb1 CTD, as an organizational center for many components of the basal transcription machinery, might preclude Rsp5 from interacting with Rpb1 during the normal transcription cycle. DNA damage may signal alterations in Pol II complexes in a manner that allows Rsp5 to recognize and ubiquitinate Rpb1. Further studies on the effects of DNA damage of Pol II holoenzyme complexes will aid in addressing this hypothesis.

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PRTB, A NOVEL PROLINE-RICH PROTEIN, IS A SUBSTRATE OF THE E3 UBIQUITIN LIGASE hRPF1/NEDD4

Maria R. Huacani and Donald P. McDonnell

Department of Pharmacology and Cancer Biology
Duke University
Durham, NC 27710

mrh@acpub.duke.edu

Previously our laboratory has identified an E3 ubiquitin ligase, hRPF1/Nedd4, as a modulator of progesterone and glucocorticoid receptor transcriptional activity. hRPF1/Nedd4 belongs to the 'hect' (homology to E6-AP at the carboxy terminus) family of E3 ubiquitin ligases which are characterized by a conserved carboxy terminal catalytic domain. While other 'hect' E3 ubiquitin ligases, including E6-AP, have been described as coactivators of nuclear receptors, we have also observed that hRPF1/Nedd4 is a potentiator of p53-dependent transcription. hRPF1/Nedd4 is likely to have many diverse cellular targets; however, as it appears to modulate two transcription factors known to play a role in breast cancer, we are interested in the identification of substrates of hRPF1/Nedd4's enzymatic activity which may explain its transcriptional effects.

Using the amino terminal substrate binding domain of hRPF1/Nedd4, we have employed a yeast two-hybrid approach to identify proteins which bind to and and serve as substrates of hRPF1/Nedd4's ubiquitinating activity. One such protein, hPRTB, is a proline-rich nuclear protein which binds to the WW domains of hRPF1/Nedd4 via a consensus WW domain-binding 'PPXY' motif. Using an *in vitro* ubiquitination assay, we have demonstrated that hPRTB is ubiquitinated by yRSP5 or hRPF1/Nedd4. Ubiquitination is dependent upon substrate binding, as a 'PY' mutant which is unable to bind hRPF1/Nedd4 is also deficient in ubiquitination. Endogenous hRPF1/Nedd4 (or perhaps a similar WW-hect E3 ligase) is able to recognize hPRTB as an *in vivo* substrate, effecting ubiquitination and degradation of wild-type hPRTB but not the 'PY' mutant in HeLa cells. Immunolocalization studies demonstrate that hPRTB colocalizes with the splicing factor, SC35, in nuclear speckles, suggesting a role for this novel proline-rich protein in RNA processing.

Thus, in our efforts to understand the mechanism by which the E3 ubiquitin ligase hRPF1/Nedd4 modulates PR and p53-dependent transcription, we have identified a novel proline-rich protein, hPRTB, as a ubiquitination substrate for hRPF1/Nedd4. We are intrigued by the possibility that hPRTB may have a role in RNA processing, as RNA processing and transcription are increasingly understood to be coupled events. It is likely that our observations linking the ubiquitination pathway and general transcription machinery will further our understanding of the complexity of transcriptional regulation.

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Identification of a Novel Ubiquitination Substrate for the Nuclear Receptor Coregulator hRPF1/Nedd4

Maria R. Huacani and Donald P. McDonnell
Department of Pharmacology and Cancer Biology
Duke University, Durham, NC 27710

In our search for proteins which modulate the nuclear receptor transcriptional response, our laboratory has previously identified the E3 ubiquitin ligase, hRPF1/Nedd4, as a modulator of progesterone and glucocorticoid receptor transcriptional activity. While additional 'hect' E3 ubiquitin ligases, including E6-AP, have been described as coactivators of nuclear receptors, the mechanism of their transcriptional effect is not well understood. E3 ubiquitin ligase proteins are the enzymes which determine substrate specificity in the ubiquitin-proteasome pathway. hRPF1/Nedd4 shares amino acid homology with the 'hect' family of E3 ubiquitin ligases, characterized by a conserved carboxy terminal catalytic domain. As little is known about the specific cellular substrates of hRPF1/Nedd4, we have sought to identify ubiquitination substrates with the goal of understanding these observations linking the ubiquitin-proteasome pathway and the general transcription machinery.

Homologs of hRPF1/Nedd4 bind and ubiquitinate several proteins from distinct cellular pathways, including RNA polymerase II and the sodium epithelial channel (ENaC). Using a two-hybrid approach, we have identified and characterized a novel hRPF1/Nedd4 substrate, human PRTB, a 17 kD proline-rich nuclear protein. hPRTB binds to the WW domains of hRPF1/Nedd4 via a consensus WW domain-binding 'PPXY' motif. We have demonstrated that hPRTB can be ubiquitinated by yRSP5 or hRPF1/Nedd4 *in vitro*. Ubiquitination is dependent upon substrate binding, as a mutant (PY), which is unable to bind to hRPF1/Nedd4, is also deficient in ubiquitination. Unlike the 'PY' mutant, wild-type hRPTB is rapidly ubiquitinated and degraded in HeLa cells. Current efforts are focused on analyzing *in vivo* interactions between hRPF1/Nedd4 and hPRTB with the goal of furthering our understanding of the complexity of E3 ligase/substrate recognition and its impact upon nuclear receptor signalling. [Supported by Department of Defense Predoctoral Fellowship, DAMD17-98-1-8072]

IDENTIFICATION OF SUBSTRATES OF hRPF1: A NOVEL E3 UBIQUITIN LIGASE

Maria R. Huacani[1], Jon M. Huibregtse[2], Sylvie L. Beaudenon[2], and Donald P. McDonnell[1]

[1]Department of Pharmacology and Cancer Biology, Duke University,
[2]Department of Molecular Biology and Biochemistry, Rutgers University

The ubiquitin-proteasome pathway is responsible for the regulation of protein stability in a wide variety of cellular processes, including gene transcription, cell cycle progression and signal transduction. E3 ubiquitin ligase proteins are the components of this multi-enzyme cascade which are believed to be key players in the selection of ubiquitination substrates. Several examples of ubiquitination dysregulation and subsequent cellular transformation have been shown to occur. The defect in these systems has been shown to occur primarily at the level of E3 ubiquitin ligase-substrate recognition. Our laboratory is interested in an E3 ubiquitin ligase, hRPF1, which was originally identified as a modulator of steroid receptor transcriptional activity. The yeast homolog of hRPF1, RSP5 has been shown to bind to and ubiquitinate the large subunit of RNA polymerase II. We postulate that there may be additional hRPF1 substrates, the identification of which will help to explain these observations linking E3 ubiquitin ligase activity and the general transcriptional machinery. Using a yeast two-hybrid approach, we have identified a pre-mRNA cleavage factor which specifically binds to and serves as a substrate for hRPF1. As RNA processing is known to be coupled to transcription, we are intrigued by the possibility that components of the RNA processing machinery might be regulated by ubiquitination. *In vitro* analysis of the regions of hRPF1 required for binding, suggest that in addition to putative ubiquitination substrates, we have also identified a class of proteins which may play a regulatory role in hRPF1 activity. Present work is aimed at determining the physiological significance of these interactions.

DEPARTMENT OF THE ARMY
US ARMY MEDICAL RESEARCH AND MATERIEL COMMAND
504 SCOTT STREET
FORT DETRICK, MARYLAND 21702-5012

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ATTENTION OF:

MCMR-RMI-S (70-1y) 17 Jun 02

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2. Point of contact for this request is Ms. Judy Pawlus at DSN 343-7322 or by e-mail at judy.pawlus@det.amedd.army.mil.

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PHYLLIS M. RINEHART
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